

Meristematic Tissues in Plant Growth and Development

*MICHAEL T. MCMANUS
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Editors*

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Meristematic Tissues in Plant Growth and Development

Edited by M.T. McManus and B.E. Veit.

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Preface

As we enter the post-genomics era, recurring themes that unify the regulation of gene expression among multicellular organisms continue to emerge. The occurrence of widely conserved molecular mechanisms across kingdoms likely reflects our common eukaryotic heritage. However, despite these similarities, sharp distinctions can be drawn between the patterns of growth and development of metazoans and those of plants. Whereas the adult body plan of an animal is established largely during embryogenesis, the body plan of a plant is elaborated throughout its life, through the activity of meristematic tissues. Although many of the patterning mechanisms that operate during embryogenesis in animal systems are becoming understood in molecular terms, the extent to which analogous mechanisms underpin the formation and function of meristematic tissues in plants remains unclear. In most cases, meristematic tissues have been described in cellular terms. Localised groups of cells whose identities are defined apparently by positional information somehow retain the capacity to divide, setting off derivatives that are destined for more differentiated, determinate fates. While these parameters have provided a convenient terminology for describing such growth, the essential nature of meristematic tissues is still not fully understood. How are stem cell populations specified in a position-dependent manner? What factors determine the predictable patterns of differentiation among their derivatives? To what extent are characteristic cellular behaviours an essential aspect of meristem function or an indirect consequence of growth processes coordinated at a supracellular level?

This volume takes a fresh look at the essential nature of meristematic tissues and the manner in which they contribute to the growth and development of the plant. Chapter 1 considers the essential features of meristems, including a discussion of how surgical approaches have contributed to our current view of the shoot apical meristem (SAM), and how these characteristics are beginning to be understood in molecular terms. The molecular mechanisms that underpin the maintenance and function of the vegetative SAM are explored in more detail in chapter 2. Chapter 3 considers the embryonic origin of the SAM, focusing on genetically defined pathways that lead to its formation. Chapter 4 provides a comprehensive review of the remarkable transformation of the SAM that occurs upon flowering, and the formation of SAMs in axillary positions is discussed in chapter 5. Chapter 6 examines the organogenic activity of the SAM, putting forward a new model for explaining the regular initiation of leaves, while the potential for meristematic-like growth during the development of the more determinate leaf is explored in

chapter 7. Chapter 8 discusses the relationship of the cell to the apical meristem, giving consideration to how their activities might be coupled. Finally, the essential aspects of the root apical meristem are examined in chapter 9, revealing intriguing parallels as well as differences when compared with the SAM.

Given the fundamental role of meristematic tissues in plant growth and development, this volume should appeal to a wide range of plant biologists, including both the specialist and the advanced student.

The editors would like to thank the individual authors of this volume for their thoughtful and timely contributions. We would also like to acknowledge the invaluable assistance of both Ann Truter in preparing the index and Joanne Allcock in preparing the final manuscript.

Michael T. McManus
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Contents

1	The organization and function of plant meristems	1
	IAN M. SUSSEX and NANCY M. KERK	
1.1	Introduction	1
1.2	The meristem concept	2
1.3	Cell formation in meristems	3
1.4	The dimensions of meristems and their domains	5
1.5	Functional domains in meristems	8
1.6	Meristems as organizational centers	10
1.7	Conclusions	13
	References	14
2	The vegetative meristem	16
	JENNIFER C. FLETCHER	
2.1	Introduction	16
2.2	Vegetative shoot meristem structure	16
2.2.1	Structural domains of the meristem	16
2.2.2	Determination of meristem cell fates	20
2.3	Maintenance of the meristematic state	21
2.3.1	Mechanisms that promote and maintain meristem activity	21
2.3.2	Non-autonomous signaling mechanisms that regulate the size and organization of meristematic tissues	28
2.3.3	Contribution of hormones to meristem maintenance	33
2.4	Organogenesis in the vegetative meristem	34
2.4.1	Regulation of organ initiation	34
2.4.2	Establishment of organ initiation sites	36
2.4.3	Establishment of boundaries between the meristem and organ primordia	40
2.5	Relation of functional domains to cytologically and anatomically defined domains	43
2.5.1	Domain-restricted gene expression patterns	43
2.5.2	Coordination between meristem domains	47
2.6	Summary	48
	Acknowledgement	48
	References	49
3.	Shoot apical meristem formation in higher plant embryogenesis	58
	MITSUHIRO AIDA and MASAO TASAKA	
3.1	Introduction	58
3.2	Morphological events during embryogenesis and SAM formation	59
3.3	Formation and maintenance of the polarity along the apical-basal axis	60

3.3.1	Origin of the apical-basal polarity	61
3.3.2	Establishment of the apical-basal polarity	61
3.4	Fate specification along the apical-basal axis	63
3.4.1	Fate specification of the embryo proper and the suspensor	63
3.4.2	Fate specification within the embryo proper	65
3.5	Elaboration of the apical region of the embryo	68
3.5.1	Formation of the SAM and boundaries between cotyledons	68
3.5.2	Cotyledon formation	70
3.5.3	Pattern formation in the apical region	72
3.6	Activation of genes involved in SAM maintenance	72
3.6.1	Function of STM in SAM development	73
3.6.2	Activation and maintenance of STM expression	73
3.6.3	Genes regulated by STM	78
3.6.4	Activation of genes involved in stem cell specification and maintenance	79
3.7	Concluding remarks	81
	Acknowledgements	82
	References	82

4. Genetic control of reproductive meristems 89

DAZHONG ZHAO, QILU YU, CHANGBIN CHEN
and HONG MA

4.1	Introduction: scope and emphasis	89
4.2	Functions and properties of reproductive meristems	90
4.3	Control of flowering time	94
4.3.1	Genes that inhibit premature reproductive development	96
4.3.2	Photoreceptors, the circadian clock and the long-day pathway	96
4.3.3	The autonomous and vernalization pathways	100
4.3.4	The gibberellic acid (GA) and abscisic acid (ABA) pathways	102
4.3.5	Integration of flowering pathways	103
4.4	Specification of floral meristem identity	105
4.4.1	The floral meristem identity genes, <i>LFY/FLO</i> , <i>API/ISQUA</i> , and <i>CAL</i>	107
4.4.2	Genes promoting inflorescence identity	110
4.4.3	Interactions between <i>LFY</i> , <i>API</i> (<i>CAL</i>) and <i>TFL1</i>	111
4.4.4	Genes controlling floral meristem determinacy	113
4.4.5	Other genes affecting floral meristem development	115
4.5	Regulation of meristem identity genes	116
4.5.1	Regulation of <i>LFY</i> expression	117
4.5.2	Regulation of <i>API</i>	117
4.5.3	Regulation of and interaction with <i>TFL1</i>	118
4.6	Control of meristem size and floral organ number	119
4.6.1	Genes promoting meristem genesis and maintenance	119
4.6.2	Control of floral organ number by meristem genes and meristem identity genes	120
4.6.3	The <i>PERANTHIA</i> gene controls the tetramerous floral pattern	121
4.6.4	Other genes affecting floral organ number	121
4.7	Interactions among and regulation of floral organ identity genes	123
4.7.1	The <i>SEP</i> genes and their role in controlling floral organ identity	123
4.7.2	Regulation of <i>AP3/PI</i> expression by <i>LFY/UFO/ASK1</i>	125
4.7.3	Regulation of <i>AG</i> expression by <i>LFY</i> and other genes	125
4.7.4	Regulation of <i>API</i> expression by <i>AG</i>	126

4.8 Conclusion and perspectives	127
Acknowledgements	128
References	128
5. Axillary meristem development	142
VOJISLAVA GRBIĆ	
5.1 Introduction	142
5.2 Branching patterns	142
5.2.1 Branching types	142
5.2.2 Evolution of branching types	143
5.2.3 Genetic control of branching types	145
5.3 Axillary meristem ontogeny	152
5.3.1 Histological approach to the study of axillary meristem ontogeny	152
5.3.2 Genetic approaches to the study of axillary meristem ontogeny	155
5.4 Patterns of axillary meristem development	157
5.4.1 Patterns of axillary meristem initiation	157
5.4.2 Patterns of axillary meristem outgrowth	161
5.5 Positional determinates of axillary meristems	163
5.6 Axillary meristem development in plants with no primary shoot apical meristem	165
5.7 Concluding remarks	167
Acknowledgements	167
References	167
6. Phyllotaxis in higher plants	172
DIDIER REINHARDT and CRIS KUHLEMEIER	
6.1 Introduction	172
6.2 Phyllotactic patterns in plants	173
6.3 Changes in phyllotaxis	175
6.4 Anatomy and function of the shoot apical meristem	177
6.4.1 The layers of the meristem	177
6.4.2 The zones of the meristem	178
6.5 Models for the regulation of phyllotaxis	181
6.5.1 Biophysical models	181
6.5.2 Biochemical models	182
6.6 Genetics of phyllotaxis	183
6.6.1 Mutants with defects in meristem initiation or maintenance	183
6.6.2 Mutants with defects in meristem organization	183
6.6.3 Mutants with defects in organ initiation	186
6.6.4 Mutants with defects in organ separation	186
6.6.5 Mutants with altered organ number or organ position (phyllotaxis)	187
6.7 Experimental evidence for models of phyllotaxis	189
6.8 Auxin regulates initiation and radial position of leaves and flowers	193
6.9 A model for the role of auxin transport in phyllotaxis	197
6.10 How does auxin regulate growth in the meristem?	200
6.11 A role for expansin and the cytoskeleton in organ initiation	201
6.12 Conclusions	204
Acknowledgements	205
References	205

7. Axial patterning and meristematic growth in leaves **213**

BRUCE VEIT and TOSHI FOSTER

7.1	Introduction	213
7.1.1	Origin of the leaf	213
7.1.2	Relationships between mechanisms controlling growth in the leaf vs stem	214
7.2	A working definition for 'meristematic tissues'	215
7.2.1	The SAM: apically limited patterns of meristematic growth	215
7.2.2	Meristematic growth in the leaf: dynamic patterns in two dimensions	215
7.3	Leaf ontogeny	216
7.3.1	The determination of the leaf	216
7.3.2	Patterns of cell division associated with the inception of the leaf primordium	217
7.3.3	Molecular markers for leaf founder identity	218
7.4	Axial patterning of the leaf	220
7.4.1	Determination of dorsiventrality	220
7.4.2	Proximal/distal patterning and morphogenesis	224
7.4.3	Lateral patterning	227
7.5	Patterns of cell division associated with lamina development	229
7.5.1	The rise and fall of the marginal meristem	229
7.5.2	Intercalary meristems	231
7.6	Regulation of determinacy	233
7.6.1	Acquisition of determinacy in leaves	233
7.6.2	Compound leaf development and intermediate states of determinacy	233
7.6.3	Promotion of indeterminacy by <i>KN1</i> -like genes	236
7.6.4	Context-dependent limitations on indeterminacy	237
7.6.5	Mechanisms by which <i>KN1</i> -like genes promote indeterminacy	239
7.6.6	Homeotic transformations in the compound leaf of pea	239
7.7	Morphology and mutants: shadows of forgotten ancestors?	244
7.7.1	'But what is the leaf': a morphological perspective	244
7.7.2	Genetic comparisons of the stem and leaf	245
	Acknowledgements	248
	References	248

8. Regulation of cell division in plants **254**

WALTER DEWITTE and JAMES MURRAY

8.1	Introduction	254
8.2	The cell cycle machinery of plants—a 'symphonic' survey	254
8.2.1	'The movements': events of the cell division and their quality control	254
8.2.2	Instruments of the orchestra and the style of conducting: cyclin-dependent kinase complexes	256
8.2.3	The symphony: cell cycle progression via the regulation of the activity of cyclin-dependent kinase complexes	265
8.3	The complete oeuvre: cell division, plant growth and development	267
8.4	Contextualisation: reflections on cell cycle regulation in the context of plant development	270
8.4.1	What is the function of the distribution of cell division?	270
8.4.2	What controls the cell cycle in the shoot apical meristem?	270
8.5	Conclusion	273
	References	273

9. The root meristem	279
PANAGIOTA MYLONA and LIAM DOLAN	
9.1 Introduction	279
9.2 Embryonic ontogeny of the <i>Arabidopsis</i> root meristem	279
9.3 Organization of cells in the primary root of <i>Arabidopsis</i>	280
9.4 Cell ablation experiments to probe determinants of cell fate of meristematic cells	283
9.5 Genetic dissection of root development	284
9.5.1 Role of auxin in the formation of root meristem in the embryo	284
9.5.2 Maintenance of the root meristem organization	286
9.5.3 Is there a correlation between cell division and cell differentiation?	287
9.5.4 The development of the radial organization of cells in the root	288
9.5.5 Specification of cell identity in the epidermis	290
9.5.6 <i>TTG</i> , <i>WER</i> and <i>CPC</i> regulate cell division	290
9.6 Perspectives	291
References	291
Index	293

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1 The organization and function of plant meristems

Ian M. Sussex and Nancy M. Kerk

1.1 Introduction

Meristems are input/output systems. The input of new cells by mitosis adds cells to the meristem and the output of cells by differentiation removes cells from the meristem. Since the processes of cell division and cell differentiation in multicellular plants are not necessarily coupled, meristems should increase in size if cell input exceeds output, and decrease in size if cell output exceeds input to the point where the meristem may cease to exist, as occurs in thorn and flower formation. Given the continual flow of cells through meristems, how are they maintained as persistent features of multicellular plants? This question has guided research in plant developmental biology for many years, and answers have depended on the technologies available at the time. The earliest studies involved observational analysis only. Later, experimental manipulations, often involving surgical, autoradiographic, and *in vitro* culture procedures were used. More recently, genetic and molecular analyses have made a great contribution to analysis of meristem organization and function. In addition, the recent focus on a few reference model systems, such as *Arabidopsis* and *Zea*, instead of a broad range of taxonomically diverse plants has resulted in a sharpening of the questions and the answers. Through repeated reinvestigation of these fundamental questions over the past century and a quarter, we have come to have a fairly detailed understanding of how meristems are organized and how they function. Needless to say, this understanding is still incomplete and will be improved as new analyses based on future technologies are developed.

Because our current understanding of meristem organization and function is built on the foundation of previous analyses, it is worthwhile to review these and discuss how they have led us to our present understanding. In this review, approaches that have been productive in analysis of vegetative phase apical and lateral meristems are discussed. However, the principal focus is on the apical meristems, which have been most thoroughly analysed and with which we are most familiar.

The present review focuses on three aspects of meristem organization and function: cell formation in meristems throughout the vegetative cycle of the plant, the overall dimensions of meristems and of their domains, and whether meristems are organizational centers for themselves and for the plant. Recent

books by Lyndon (Lyndon, 1998) on the shoot apical meristem and by Larson (Larson, 1994) on the vascular cambial meristem extend some of the views presented here, as do other chapters in this volume. References to much of the research on plant meristems carried out before about 1987 can be found in Steeves and Sussex (Steeves and Sussex, 1989).

1.2 The meristem concept

Meristems, defined as localized sites of continued cell division, are characteristic of most multicellular plants. Unicellular plants alternate between a phase of cell division and a phase of mature cell function, such that each cell is meristematic at one time in its life history. A few simple multicellular plants, such as the algae *Spirogyra* and *Ulva*, display a similar growth pattern, but in all other multicellular plants, meristems are localized at specific sites in the plant body (Graham *et al.*, 2000). The advantage of such a body plan is that the meristematic zones, in which cells are forming, specializing and differentiating, are spatially separated from the functional zones of mature cells, in which photosynthesis, movement of major organic and inorganic molecules and synthesis of cellular components and secondary compounds is occurring. Thus, these plants are able to grow continuously while maintaining full metabolic activity (Niklas, 2000; Sussex and Kerk, 2001).

The spatial separation of meristems from mature cell zones is reflected in meristem nomenclature. Apical meristems, the shoot apical meristem (SAM) and the root apical meristem (RAM), are located at the tip of each shoot and root axis, respectively, in vascular plants. An apical meristem is also at the tip of each axillary shoot and each lateral root. An apical meristem is also located at the tip of each major axis in bryophytes and some brown algae, such as *Fucus*.

Cells differentiating from apical meristems contribute to length growth of the axis by progressing through a succession of developmental states, in which cells continue to divide for some time, enlarge and undergo differentiation. These regions of mitotically active, apical-meristem-derived cells are sometimes termed primary meristems and they are then classified as dermal, ground and vascular, according to the tissues that mature from them.

Intercalary meristems are regions, often at the base of an internode or of a monocotyledonous leaf, where mitosis persists transiently after it has ceased in more proximal and distal cells. The vascular cambial meristem (VCM) and cork cambial meristem (CCM), the latter also referred to as phellogen, are lateral meristems that occur near the periphery of shoot and root axes. Their cell-forming activities result in growth in girth of the axis. Sometimes, the apical meristems of lateral shoots and roots are referred to as lateral meristems. However, in the present text this term will be used to refer to the VCM and CCM.

Although meristems are frequently contrasted with their differentiating derivatives as regions of undifferentiated cells, this concept is questionable. While it may be relatively simple to define a differentiated cell based on its cytology, histology or specific gene products, the concept of 'undifferentiated' is not supported by objective criteria. The cellular characteristics of the SAM, RAM, VCM and CCM are each distinctive, indicating that the cells in these four meristem types have progressed along different developmental pathways. Using similar arguments, meristems cannot be referred to as developmentally undetermined as a contrast to their determined products, because these meristems are already determined as to the organ and tissue types that they produce. Furthermore, SAMs and RAMs are not developmentally interconvertible. Indeed, there may be no undifferentiated plant cell, since even the zygote is affected by its association with the maternal cellular environment (Ray *et al.*, 1996). It may be simplest, therefore, to recognize that the developmental states of meristems are different from one another and from those of their derivative cells.

1.3 Cell formation in meristems

The axes of mosses, such as *Polytrichum*, terminate in a single, large apical cell. Shoots and roots of *Equisetum* and ferns, such as *Osmunda* and *Azolla*, also terminate in a large, often tetrahedral, apical cell that divides in predictable planes to produce merophytes, from which all cells in the axis can be traced anatomically. These observations led to the suggestion that a similarly positioned cell or cell group might be the progenitor of all cells in the axes of gymnosperms and angiosperms. Although numerous investigations have failed to detect enlarged cells in predicted positions, cells at the summit of these meristems have been observed in mitosis, supporting the idea that this was the location of the progenitor cells of the organ. In early reports, progenitor cells were referred to as initial cells. However, controversy arose as to how initial cells might be identified, how many initial cells each meristem contained and whether they were permanent or not. Thus, the term progenitor cells, referring to the cell population, seemed to be preferable at the time.

A contrary opinion as to the location of progenitor cells emerged from observations made by workers who failed to detect mitotic activity in the distal cells of vegetative shoot meristems of the gymnosperms and angiosperms that they investigated. They proposed that cells at the summit of the SAM were mitotically inactive during post-embryonic vegetative development but became mitotically reactivated to produce the reproductive meristem. This region of the SAM was named the *méristème d'attente* (Buvat, 1952).

At approximately the same time that these observations were being made on the SAM, the same issue emerged from studies of the RAM in angiosperms. Attempts to demonstrate surgically, or by ^3H -thymidine labeling, that the cells

of the *Zea mays* (maize) root originated from a small group of progenitor cells located near the tip failed to identify such a mitotically active cell group. Instead, cells in this region of the RAM appeared to be mitotically inactive and this region was named the quiescent center (QC) (Clowes, 1956).

How could it be that studies designed to identify mitotically active progenitor cells at the summit of the meristem of shoots and roots instead found mitotically inactive cells at these locations? The answer to this question was obtained through studies that paid more attention to division frequency and cell cycle times. There is no reason, *a priori*, why cells that are the progenitors of the axis would be expected to divide more frequently or as frequently as the cells that they give rise to. As long as the presumed progenitor cells cycled slowly to produce derivative cells that cycled sufficiently rapidly to produce the cells that generated the organ, the distal cells would be the progenitors.

A clear example that illustrates this concept was described in the shoot apex of *Ligustrum*, where clonal analysis indicated that the distal cells divided only once during the formation of three or four phytomers, the node-internode units of the stem. The calculated cell cycle time of the progenitor cells was 12 days (Stewart and Derman, 1970). This observation was consistent with many observations of variations in cell cycle times within SAMs. These showed that cells at the summit divide less frequently than those on the apical flanks. This was also observed in the roots of maize, in which study of cell cycle times indicated that, whereas cells in the proximal and distal meristem zones that surround the QC divided on average every 12–24 h, cell cycle time in the QC was approximately 144 h or more (Clowes, 1961).

An additional consideration is that many of the earlier studies on division patterns in meristems did not consider the meristem throughout development. These studies came to conclusions that, while valid for the period of observation, failed to account for the totality of the division pattern. For example, in the perennial long roots of *Euphorbia esula*, the QC is well developed at the height of the growing season, but early in the season, as root growth is being reactivated, no QC can be detected and cells are dividing in this region (Raju *et al.*, 1964). The apical cell in *Azolla* roots is also mitotically active early in root growth. However, these roots are determinate and later in development the apical cell fails to divide mitotically and becomes endopolyploid. Until this biology of the root was taken into account, contradictory analyses were published (Gifford, 1983).

It is now generally accepted that what have been called ‘the ultimate initials’ within the SAM and RAM of all vascular plants are located at the summit. More recently, these cells that are the progenitors of the entire organ have been named ‘stem cells’, in conformity with the terminology from animal developmental biology.

From the studies reviewed above and many others like them, we now have a better appreciation of the significance of cell formation in apical meristems.

and the variation of cell cycle times in different regions of vegetative apical meristems at different times in the growth cycle. A question that has emerged from these studies is whether cell cycle time is important for organ morphogenesis. For example, on conversion of a vegetative SAM to a reproductive meristem, whether inflorescence or flower, cell cycle times become shorter and may become more uniform in duration and synchronized throughout the meristem. However, the significance of this phenomenon is unclear (Lyndon, 1998).

Analysis of cell formation in the vascular cambial meristem has been complicated by differing definitions of the meristem and by the presence therein of two cell types, fusiform initials and ray initials. During periods of active growth, there is a band of dividing and differentiating cells called the cambial zone between mature secondary phloem and secondary xylem. Each radial cell file, from the exterior to the interior, consists of phloem mother cells, a cambial initial and xylem mother cells. Autoradiographic analysis using ^3H -thymidine as a marker for DNA synthesis found that mitoses are relatively frequent in the differentiating xylem and phloem mother cells and less frequent in the region of cambial initials. Thus, as in apical meristems, the progenitor cells in the cambium have a reduced frequency of mitosis (Gahan, 1989).

A general conclusion that emerges from the above studies is that the stem cell population in different kinds of meristems has a lower frequency of mitosis than derivative cells do. What is the significance of this finding? It has been speculated that because damage to DNA occurs preferentially in the S-phase of the cell cycle, by reducing the number of cell cycles in the stem-cell population, the genetic integrity of the plant and the gametes that are derived from the SAM stem cells can be conserved. How the differences in cell cycle times in the different regions of meristems are controlled at the molecular level remains unclear (Doonan, 2000). A biochemical model for regulation of mitosis in the QC of the maize root has been proposed, in which polar transport leads to the accumulation of auxin in the root tip. Elevated auxin levels cause upregulation of expression of the ascorbic acid oxidase (AAO) gene, and increases in AAO protein accumulation and enzyme activity. Ascorbic acid, which is required for progress from G1 to S in the cell cycle, is metabolized, resulting in very low levels of mitotic activity in the QC (Kerk and Feldman, 1995).

1.4 The dimensions of meristems and their domains

In this section, studies related to two questions concerning meristem size are analyzed. First, is there a precise boundary between a meristem and its differentiating cellular products, and second, are there domains within meristems that have specific functions? If so, how are these domains maintained in the face of cell flow through the meristem?

It is important to be able to identify the boundary between meristems and the cells derived from them. Within the meristem, cells are pluripotent, whereas those outside the boundary are destined for differentiation that restricts their further developmental potential. Knowledge of the molecular differences between pluripotent meristem cells and differentiating cells would allow us to probe more deeply into the processes of cell differentiation. However, designating meristem boundaries is not always a simple matter, and we need to consider whether there is a strict boundary or a gradual transition in properties between meristem cells and their differentiating products.

In plants where a single apical cell is defined as the meristem in a shoot or root, and derivative cells are produced as merophytes that proceed through an apparently stereotyped sequence of divisions to contribute a unit to the mature structure, the meristem boundary seems to be easy to define. However, there is no experimental verification that persistent meristem function is limited to a single cell, and we do not know what the consequences would be if the apical cell was destroyed. However, in plants with multicellular meristems, the cytological and histological distinction between cells that lie within the meristem's presumed boundary and those that lie just outside it may be minimal, and other criteria are needed to define the boundary. One of the first workers to address this question critically was Wardlaw, who investigated the organization of the SAM of the fern, *Dryopteris dilatata* (Wardlaw, 1949). He defined the SAM as consisting of the enlarged apical cell and its immediate derivatives that were produced by successive anticlinal divisions to form a surface layer of prism-shaped cells. The boundaries of the meristem were marked by divisions of the prismatic cells. Peripherally, they divided periclinally several times and the derivative cells differentiated as epidermis and cortex. Internally, the small basal cells cut off by asymmetric division of prismatic cells differentiated as vascular tissue and pith. Thus, the SAM was considered to consist of a single layer of readily identifiable cells.

This definition is not applicable to the SAM of angiosperms, where the cellular organization of the meristem is quite different. In these meristems, the surface layer of cells (the L1 layer) divides anticlinally both within the meristem and in differentiating derivatives which mature as epidermis. The underlying layer of cells (L2) divides anticlinally within the meristem, but usually undergoes a succession of periclinal divisions on the flanks of the apex (Sussex, 1955). The boundary between anticlinal and periclinal divisions in L2 was used to identify the lateral boundary of the meristem of potato. That this boundary had developmental significance was shown by an experiment, in which a small segment of the shoot tip was surgically separated from adjacent regions by a vertical incision. If the incision was on the SAM side of the anticlinal/periclinal border so as to leave a band of meristem cells outside the incision, and the rest of the SAM was surgically removed, the meristem band reorganized into a functional SAM. If the incision was made at the anticlinal/periclinal border

and the entire SAM was removed, then the cells outside the incision failed to reorganize as a meristem and continued to differentiate as expected (Sussex, I.M., unpublished).

This experiment supports the concept that the developmental fate of cells lying within the SAM is quite different from that of cells lying immediately outside it, and supports the view of a definite meristem boundary. Within the past few years, support for this view has accumulated rapidly as molecular analyses of meristem organization have become more detailed. Several genes are now known to have expression patterns that are restricted to cells of the SAM, and these are not expressed in differentiating cells. The lateral boundary of expression of these genes thus marks the lateral boundary of the meristem. An example is the *Arabidopsis* gene, *CYP78A5* (Zondlo and Irish, 1999), where expression is limited to a region on the meristem flanks and does not extend beyond this. The expression pattern of *BLEC*, a lectin-like gene of pea, is the reciprocal of that of *CYP78A5*. *BLEC* RNA is not expressed within the SAM but is expressed in L1 cells immediately outside the meristem (Maiti *et al.*, 1993).

While defining the lateral boundary of the SAM appears to be amenable to resolution by a combination of histological, experimental and molecular approaches, the location and characteristics of the internal boundary between the meristem and its basal derivatives is more problematic. The SAM of gymnosperms and angiosperms has been defined by the cytohistological characteristics of different zones within it. A general definition is of a distal central zone, a lateral peripheral zone, and a basal rib or file zone. With some variations of terminology, this description of the SAM is widely accepted for gymnosperms and angiosperms (Steeves and Sussex, 1989). When the vertical depth of the meristem is examined using cytohistological criteria, it has been found to consist of many layers of cells, in some cases exceeding eight or nine. However, no attempts have been made to determine the basal boundary of the meristem using criteria similar to those used to define the lateral boundary. Different analytical procedures suggest that the vertical extent of the angiosperm SAM may be much less than cytohistological criteria indicate. In periclinal chimeras, it is possible to trace the derivation of all of the differentiated cells in the shoot to the SAM cell layer that generated them. By analyzing various polyploid periclinal chimeras, Satina and colleagues demonstrated that the SAM of *Datura stramonium* consists of three superposed layers that they named L1 (surface), L2 and L3 (deepest) (Satina *et al.*, 1940). Within the SAM, L1 and L2 layer cells divide only anticlinally but L3 cells divide in various planes. These three layers corresponded well in their proposed function with tunica corpus terminology, by which *Datura* was considered to have a two-layered tunica overlying the corpus. Analyses of periclinal chimeras in other species have indicated that the three-layered SAM (L1, L2 and L3) is relatively common, but this is not a universal condition. In oat, wheat, rye and many other monocotyledonous plants, there is a single surface layer in the meristem that by anticlinal and periclinal divisions

generates the entire organ. The stem cells that are the progenitors of all the cells in the shoot are located distally within these cell layers.

The conclusion to be drawn from these studies of chimeras is that in its genetic behavior the SAM is much shallower than histological analysis had suggested. What are we to make of this discrepancy? Are the deeper cell layers, described histologically as rib or file meristem, not part of the SAM but cells that are in early stages of differentiation? One way to examine this question is to identify genes that are expressed in deeper cell layers of the shoot tip and determine whether their expression is required to maintain organization and function of the meristem. In other words, is the function of these genes so integral to the maintenance of meristem function that the cells in which they are expressed should be considered as essential components of the meristem?

An *Arabidopsis* gene with such an expression pattern is *WUSCHEL* (*WUS*). *WUS* is expressed in a small number of L3 cells but not in the uppermost L3 layer. *WUS* induces expression of *CLAVATA1* (*CLV*) and 3 in more superficial cells, and this has been used to define these as the stem cells of the meristem (Schoof *et al.*, 2000). *WUS*-expressing cells, therefore, are considered to act as the organizing center of the SAM. It seems reasonable to conclude that *WUS*-expressing cells are essential components of the meristem, and that they represent its basal boundary. This interpretation of organization of the SAM means that the file meristem within which *WUS* is expressed is a domain within the meristem.

1.5 Functional domains in meristems

The SAM can be subdivided into cytohistologically distinct zones or domains, which have distinct functions and distinct patterns of gene expression. The central zone is the location of stem cells, and the peripheral zone is where organogenesis commences. It is interesting to question whether cells in the peripheral zone are already irreversibly committed to organogenesis and cellular differentiation, or whether they still retain the pluripotential capacity of stem cells. This question has been addressed by surgical and *in vitro* culture procedures, and more recently by molecular analysis of *Arabidopsis* mutants.

Needle punctures into the SAM summit of *Dryopteris* and potato resulted in the formation of new meristems from the flanking cells of the meristem (Sussex, 1964). When regions of the peripheral zone were surgically isolated from the central zone or were grown *in vitro*, they developed as fully organized meristems with typical histological organization (Ball, 1960). These experiments demonstrated that the peripheral zone cells are not irreversibly committed to organogenesis and differentiation, but retain the capacity to express the pluripotent features of stem cells, although this is not expressed in normal development. Thus, the peripheral zone cells must detect their position in the meristem

relative to the stem-cell-containing central zone and function accordingly. When the presumed central zone signals are removed by surgical intervention, the peripheral zone cells resume central zone function.

More recent experiments with *Wuschel* mutants of *Arabidopsis* also support this conclusion. In these mutants, activity of the SAM terminates soon after germination, and the center of the meristem is occupied by vacuolated and partially differentiated cells. Subsequently, new meristems and leaf primordia are initiated from peripheral regions and also across the summit of the apex. These secondary meristems repeat the behavior of the original meristem, so that mutant plants develop in a 'stop-and-go' mode (Laux *et al.*, 1996). Since *WUSCHEL* expression is required to maintain stem cell function in *Arabidopsis* SAMs, we can conclude that lack of stem cells in *wus* mutants results in failure of stem-cell-to-peripheral-zone signaling and that peripheral cells then resume stem-cell function.

These surgical and genetic experiments support the conclusion that the domains of specific function and gene expression in the SAM are maintained by signaling between different regions within the meristem. But what is the nature of the signals? As discussed further in chapter 2 of this volume, genetic and molecular analysis of gene expression in the SAM of *Arabidopsis* supports a biochemical interpretation of signaling, in which transcription factors, protein ligands, and receptors play important roles. However, there is another possibility, and that is that meristem domains are established and maintained, in part at least, by mechanical forces, and that the distribution of forces differs in the central and the peripheral zone (Green, 1999). Analysis of surgical incisions made in the inflorescence meristem of sunflower has indicated that the central zone is under radial and circumferential tension, but the peripheral zone is under a compressive circumferential force and radial tension (Dumais and Steele, 2000). This difference in stress distribution might determine the central zone/peripheral zone boundary directly, or might act indirectly by regulating the expression of genes that respond to mechanical stress, or by determining the orientation of cytoskeletal elements and the planes of cell division within the meristem domains.

The regulatory loop between *CLAVATA* and *WUSCHEL* genes may also form the basis for understanding the dynamic changes in size that the meristem and its domains undergo during development. These changes include: the alternation between maximal and minimal volumes associated with plastochronic leaf initiation, the ontogenetic increase in meristem volume (which approaches ten per cent in each plastochron in maize), the usual increase in volume in the transition to reproductive development, and the seasonal fluctuation in meristem volume in perennial plants. In wild-type *Arabidopsis* SAMs, *WUS* positively regulates the expression of *CLAVATA1* and 3. Products of these genes repress lateral expansion of *WUS* expression in deeper layers of the meristem. In *clv* mutants, *WUS* expression expands laterally, resulting in a greatly enlarged

meristem with a large central zone (Brand *et al.*, 2000). A possible mechanism for the changes in meristem dimensions observed during development is that the regulatory loop between *WUS* and *CLV* genes is subject to periodic modification by as yet undiscovered genes that alter the levels of expression or the interactions between these genes.

Less attention has been paid to functional domains in the RAM. However, within this meristem, regions with distinct functions can be identified and these are described more fully in chapter 9 of this volume. The QC in maize consists of about 600 cells, whereas in *Arabidopsis* it can be as few as four cells. Surrounding the QC are domains where the cell cycle times are shorter than in the QC. In maize, these domains are the proximal meristem, whose derivatives differentiate as cells of the root axis, and the distal meristem, whose derivatives differentiate as root cap and epidermis. In *Arabidopsis*, comparable domains are referred to as initial cells, although this term is used differently here when compared with the SAM.

1.6 Meristems as organizational centers

Meristems are the source of cells that generate plant structure. But are the meristems organizing centers for themselves and their derivatives? Is the information that is required for continued meristem function and the differentiation of derivatives located within the meristem, as suggested by the *WUS-CLV* loop? An alternative hypothesis is that meristems are centers of cell production and nothing more than that. The information required for meristem function and for tissue and organ initiation originates outside the meristem. A third hypothesis is that some information is endogenous to the meristem and some is exogenous, and that different meristem types differ in this regard. Approaches to these questions have changed as new technologies became available. Early studies were observational only. Later, experimental manipulation designed to interfere with the meristem/derivative relationship was employed. More recently, mutant and molecular analyses have contributed to our current understanding of these questions.

If meristems are autonomous in their function, they should continue to develop normally when separated from the remainder of the plant if supplied with basic nutrients. This is the definition of autonomy that will be used here. This experiment has been performed in numerous studies on apical and cambial meristems, with quite consistent results. That is, the SAM and RAM possess a high degree of autonomy although not perhaps complete, whereas the VCM is not autonomous. Thus far, no experiments have tested the CCM.

Early experiments on apical meristems involved separating the SAM from laterally contiguous tissue by vertical incisions, leaving it supported on a plug of developing pith. In *Dryopteris* and in lupin, meristems isolated in this way

continued to develop, giving rise to tissues and organs that were normal in position and histology. As knowledge of the nutrient requirements of plant cells improved, SAMs and root tips were excised entirely from the plant and grown in sterile culture. In a variety of fern and angiosperm species, the excised meristems continued to function in a phenotypically normal fashion forming tissues and organs. In the fern experiments, nutrient media could be as simple as sugar and inorganic salts, but for angiosperms addition of B vitamins, an auxin and a cytokinin was usually necessary to obtain continued development (Steeves and Sussex, 1989).

In these *in vitro* experiments, although the explants were quite small, in some cases being less than 0.1 mm^3 , it is questionable that they consisted of the apical meristem devoid of all derivative, partially differentiated cells. For example, if the fern SAM consists of a single superficial layer of cells, then the explants which contained several cell layers clearly contained more than the SAM. Similarly, given the difficulty of determining the basal boundary of the angiosperm SAM, explants may have contained some partially differentiated cells that could have provided materials essential for continued development of the meristem. Likewise, excised root tips contained, in addition to the RAM, the root cap and some differentiating cells. One experiment, however, appears to have established the degree of autonomy of the RAM of maize unequivocally (Feldman and Torrey, 1976). These workers separated the QC from surrounding cells and, using a culture medium that contained an organic nitrogen source, auxin and kinetin, in addition to sucrose and inorganic salts, the QCs were observed to produce roots of normal morphology that preserved the polarity of the original explants.

These experiments demonstrated that, provided basic nutrient requirements are satisfied, many SAMs and RAMs continue to develop more or less normally when detached from the plant. Others require the addition of growth regulatory molecules and B vitamins, and there is the question of possible inclusion of some differentiating cells in the explant with their presumed different biochemistry. For example, when SAMs of *Dianthus* were excised without any leaf primordia, they failed to develop in culture even when the medium was supplemented with inositol and thiamin (Shabde and Murashige, 1977). Most of the meristems isolated with two pairs of leaf primordia and the youngest pair of expanding leaves developed into complete rooted plants on this medium. When the medium was further supplemented with indole-3-acetic acid and kinetin, most of the meristems isolated without leaf primordia also developed into complete plants. This experiment suggests that hormones required for SAM development are synthesized in young leaves and transported to the meristem. From the experiments discussed here, it is possible to conclude that, at the very least, the SAMs and RAMs that were tested display a high degree of autonomous function.

Another way to approach the question of autonomy of the SAM is through analysis of its response to altered environmental conditions. Excised SAMs of

Thlaspi growing in culture became vernalized and produced flowers after having been exposed to low temperatures, indicating that the vernalization signal can be perceived by the meristem itself (Metzger, 1988).

When experiments comparable to excision and *in vitro* culture of apical meristems were carried out on the VCM, the results were quite different. The isolated VCM did not function autonomously to produce normal derivative tissues. The experiments involved separating pieces of the meristem and some derivative cells from the plant and culturing them *in vitro*. These explants consistently proliferated as callus, in which elongated cambial fusiform initials could not be identified.

In the plant, the VCM is physically constrained between differentiated secondary xylem and secondary phloem, and is presumably subject to increased radial compression as the diameter of the axis increases during growth periods. That physical pressure may be a factor in maintaining organized structure and function of the VCM has been demonstrated in an experiment where pieces of the cambial zone and adjacent secondary phloem of *Populus deltoides* were cultured *in vitro* and subjected to compressive forces from a mercury column. The cambial fusiform initials maintained their identity and produced derivative cells that differentiated as vascular elements (Brown, 1964).

The previous experiments have revealed a high degree of organizational autonomy in apical meristems. But are meristems also the organizing centers for the cells derived from them? That is, does information from the meristem determine patterns of tissue differentiation? Many of the observations and experiments that bear on this question are inferential at best. However, there is some evidence that the meristem determines some differentiation patterns, but not others. The fact that new apical meristems can be initiated in callus cultures and produce phenotypically normal organs has been taken as evidence that meristems determine the patterns of differentiation of their products. More direct evidence comes from the following experiment. The SAM of carrot was isolated on a plug of pith tissue, and newly forming leaf primordia were destroyed as they appeared (Xia and Steeves, 2000). In the newly formed axis, differentiation of vascular tissue proceeded only to the provascular stage. If a single leaf primordium was left to develop, vascular differentiation proceeded normally in the axis below it and mature xylem and phloem cells differentiated. Thus, the SAM apparently determines the initial differentiation of provascular cells, but their maturation as xylem and phloem requires a leaf-derived stimulus, which may be auxin.

In the root it has been proposed that the QC functions as an organizing center for patterning the root tip. In an experiment where the root cap and QC of maize were removed, tissue differentiation in the stump was arrested and resumed only after a new QC had organized, supporting the primary role of the QC in tissue patterning (Feldman, 1976). But there is some evidence that information directing cell differentiation also originates in older cells and is transmitted to

younger cells to control their differentiation. The QC of *Arabidopsis* inhibits differentiation of contiguous cells in the meristem, maintaining them as initial cells that give rise to daughter cells with specific developmental fates. Laser ablation of individual QC cells resulted in differentiation of the contiguous initial cells, indicating that the QC is a negative regulator of differentiation in these cells (van den Berg *et al.*, 1997). Thus, the QC functions to maintain the self-renewing properties of surrounding initial cells of the proximal and distal meristems. But the fate of cells derived from division of initial cells appears to be determined by signals derived from more mature cells. This was shown by laser ablation of daughter cells of the cortical initials (van den Berg *et al.*, 1995). Each daughter cell normally undergoes a periclinal division to form an endodermal cell and a cortical cell. But this division only occurred when the daughter cell was in contact with an older cell in the same file after the ablation. This suggests that the older cell acts as a patterning template for the definitive division of the younger cell into endodermal and cortical cells.

1.7 Conclusions

This review of the historical development in our understanding of the organization and function of plant meristems reveals a remarkable consistency in interpretation as technologies have changed. The early observational studies, the experimental manipulations and the recent genetic and molecular analyses have all contributed to our current understanding. Particularly surprising, in the face of criticisms of surgical approaches for their supposed generation of wound hormones, including ethylene, is the evidence that these approaches have provided for functional domains and for the interdependence of these meristem domains—this has been confirmed and extended by molecular analyses.

Another surprise that has come from genetic and molecular studies is the different control systems that appear to distinguish SAMs from RAMs. Mutations that affect one of these meristems do not usually affect the other. This indicates that the molecular controls for function are specific to the particular meristem type. This conclusion is strengthened by failure to detect the presence of SAM gene products in the RAM, for example. Given that primitive vascular plants were rootless and that the root is considered to have evolved by modification of shoot structures, this suggests a great degree of evolutionary divergence between the SAM and the RAM.

Little is said about the lateral meristems in this overview. Clearly they are of great economic importance and merit attention. An interesting fact is that lateral meristems have evolved independently on several occasions, for example in *Lepidodendrids*, *Calamites* and seed plants, and so molecular comparisons would be of value but are impossible due to extinctions.

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2 The vegetative meristem

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2.1 Introduction

The vegetative shoot apical meristem is a highly organized yet dynamic structure. It is responsible for maintaining the proliferation of a population of undifferentiated stem cells throughout vegetative development, as well as for initiating lateral organs in stereotypical patterns. The vegetative shoot apex must maintain a continuous balance between the production of stem cells and the incorporation of their derivatives into organ primordia, as either excess accumulation or excess loss of meristem cells has severe morphological consequences for the plant. This chapter reviews our current understanding of four major aspects of vegetative meristem function: how the meristem is organized, how the meristematic state is maintained throughout vegetative development, how patterned organogenesis is initiated, and how the cytological and anatomical domains of the vegetative meristem correspond to functional domains.

2.2 Vegetative shoot meristem structure

2.2.1 Structural domains of the meristem

The shoot apical meristem is composed of a relatively small population of cells at the growing tips of the plant, yet is the source of all of the above-ground organs. The size of the vegetative meristem varies widely throughout the plant kingdom, ranging from 3.5 mm in diameter in some cycad species down to 50–80 μm in *Arabidopsis thaliana* and other angiosperms (Steeves and Sussex, 1989). Vegetative meristem geometry and cell number can also vary between different inbred backgrounds or ecotypes within a single species. Measurements of meristem size in *Zea mays* reveal significant height and width differences between inbred lines (Vollbrecht *et al.*, 2000). Similarly, the number of shoot meristem cells in the *Arabidopsis* Landsberg *erecta* ecotype at germination has been estimated at approximately 110 (Irish and Sussex, 1992), while the number in the Wassilewskija ecotype is approximately 70 (Medford *et al.*, 1992). The morphology of the vegetative shoot apex is also variable, ranging from domed or elliptical to flat or even slightly depressed (Evans and Barton, 1997). Changes in morphology can also be observed in many species depending upon the stage of

development of the plant, the time of year and the involvement of the meristem in initiating leaf primordia.

The vegetative meristem of plants typically consists of a small dome of proliferating stem cells that has specific structural features (figure 2.1). In angiosperms, one level of organization, established in the embryo, is the stratification of cells across the meristem into layers (Satina *et al.*, 1940; Poethig, 1987). One or more layers of the shoot apex that display predominantly anticlinally oriented cell divisions are referred to as the tunica, and the underlying region of cells that divide in all planes is termed the corpus (Schmidt, 1924). One to five tunica layers are observed among angiosperm species, with two layers being the norm in dicotyledonous plants and one in grasses (Esau, 1965). The number of tunica layers is static in most species, but can vary in relation to developmental stage, to the timing of lateral organ initiation, and, in perennial plants such as *Daphne pseudomezereum*, to seasonal changes in growth (Steeves and Sussex, 1989).

In *Arabidopsis* and most other dicotyledonous plants, the tunica consists of an overlying epidermal L1 layer and a subepidermal L2 layer (Gifford, 1954). Each layer is a single cell thick, and each remains clonally distinct from the others by continuous anticlinal cell division (Tilney-Bassett, 1986). The corpus, or L3 layer, lies below the tunica and consists of cells that divide both anticlinally and periclinally. Clonal analysis using periclinal chimeras indicates that in dicotyledonous plants, such as *Arabidopsis*, the L1 cell layer derivatives

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Figure 2.1 Histology of the *Arabidopsis* shoot apical meristem. A: The meristem central zone (CZ) lies at the apex of the meristem, with the peripheral zone (PZ) towards the flanks and the rib zone (RZ) beneath. The central zone replenishes both the peripheral zone and rib zone (arrows) and also maintains the integrity of the central zone itself. B: The shoot apical meristem is composed of clonally distinct layers of cells. The epidermal layer (L1) and the subepidermal layer (L2) constitute the tunica, while the L3 layer makes up the corpus. *FILAMENTOUS FLOWER (FIL)* mRNA expression (dark, wedge-shaped patch) demarcates the lateral organ anlagen in the meristem peripheral zone. (Reprinted from Eshed and Bowman, 2000. Formation and maintenance of the shoot apical meristem, *Trends in Plant Science*, 5 110-115, with permission from Elsevier Science.)

give rise to the epidermis of shoots, leaves and flowers, while the L2 cell layer provides the mesodermal tissue and the germ cells found in pollen grains and ovules (Tilney-Bassett, 1986). Derivatives of the L3 cell layer include the pith and vascular tissues of the stem, and the most interior cells of leaves and floral organs. In addition to their clonal isolation, the layers of the *Arabidopsis* meristem are distinguishable based upon differences in cell size, with a gradient of increasing cell diameter observed from the outer L1 to the inner L3 cell layer (Laufs *et al.*, 1998b). In monocotyledonous plants, such as maize, the tunica is a single cell monolayer corresponding to the epidermis, with the corpus making up the internal tissues (Steffensen, 1968).

Both the tunica and the corpus participate in meristem maintenance and organ formation (Poethig and Sussex, 1985a and b), and meristem growth and cell fate specification during development are coordinated between all the cell layers. This has been demonstrated by Satina and colleagues, who generated mosaics of the otherwise diploid thorn apple, *Datura stramonium*, in which the epidermal layer was tetraploid or octaploid (Satina *et al.*, 1940; Satina and Blakeslee, 1941). In both cases, growth and development of the meristem occurred normally despite the greatly enlarged epidermal cells. Cell proliferation was markedly reduced in the epidermal layer of these mosaic meristems, so that the area of the L1 layer matched that of the underlying layers. Similar compensation occurred if the polyploid cells were present in one, or more, of the other cell layers (figure 2.2). Thus, the regulation of meristem growth can be independent of cell number, and an increase in cell size in any one layer of the meristem can be sensed and compensated for by corresponding changes in cell proliferation.

The organization of the tunica-corpus reflects specific patterns of cell divisions within the angiosperm vegetative meristem, with the anticlinal divisions of the tunica layer(s) contributing to surface growth and the variable division orientations of the corpus providing growth by volume (Esau, 1965). However, this concept of meristem organization has proved less suitable for the characterization of gymnosperm shoot apices, as only a few gymnosperms, such as *Ephedra* and *Gnetum*, appear to have an overlying, clonally distinct stratus that

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Figure 2.2 Coordination of meristem growth independent of cell number in plants. Sections through the shoot apex of chimeras in *Datura*. A: Layers all diploid; B: outer layer octoploid, inner layers diploid; C: outer layer diploid, subepidermal layer octoploid, inner layers diploid. (Reprinted from Day and Lawrence, 2000. *Development*, 127 2977-2987, with permission from the Company of Biologists Ltd.)

can be interpreted as a tunica layer. In many other gymnosperm species, the outermost cells can divide periclinally, and thus do not remain clonally distinct from the underlying cells (Johnson, 1951). In general, the shoot apices of lower vascular plants also are not stratified. Instead, they usually consist of an apical initial cell that divides anticlinally, while the other surface cells divide both anticlinally and periclinally. In ferns, the periclinal divisions are unequal, such that the smaller daughter cell is interior and the larger derivative retains the distinctive morphology of the surface cells (Steeves, 1963). Despite its limited application across the plant kingdom, the tunica-corpus concept is nonetheless useful for describing how specific cell division patterns organize the apices of higher plants, and for tracing the derivation of the different tissues from meristem precursor cells.

Cytological and histological studies also indicate that the vegetative meristem consists of distinct radial domains (figure 2.1). Examination of median longitudinal sections through the shoot apex of the ancient gymnosperm, *Ginkgo biloba* (Foster, 1938), provided early evidence for a radial organization to the vegetative meristem. At the very apex of the *Ginkgo* meristem lies a cluster of enlarged, highly vacuolated cells that divide infrequently relative to the other cells in the meristem. Foster designated this cluster the central mother cells. Surrounding the central mother cells laterally and basally, like a collar, is a region consisting of small, densely cytoplasmic cells. These cells were termed the peripheral subsurface layers. Beneath the zone of central mother cells, in the deepest layers of the meristem, resides a series of rows or columns of small, vacuolated cells referred to as the rib meristem. Between the zone of central mother cells and the other regions is a transition zone, in which the characteristics of the central mother cells are gradually replaced by those of the surrounding zones.

This general pattern of cytohistological zonation has been found to be typical of both gymnosperms and angiosperms. A pattern of radial organization similar to that of *Ginkgo* was first described for the *Heracleum* shoot apex (Majumdar, 1942), and has subsequently been observed in *Helianthus* (Davis *et al.*, 1979) and *Arabidopsis* (Medford *et al.*, 1992), among other flowering plants. While the cells at the summit of the central mother cell cluster have also been referred to as the apical initials and those beneath them the subapical initials, these terms have gradually been replaced by the term 'central zone'. Like their gymnosperm counterparts, the cells of the angiosperm central zone divide infrequently and have a weak affinity for histological stains for either the nucleus or cytoplasm (Medford *et al.*, 1992). The central zone cells are surrounded by a peripheral zone of small, densely staining cells that divide more frequently, and overlie a rib zone of rapidly dividing cells that contribute to the meristem pith (Steeves and Sussex, 1989). From measurements of mitotic activity in more than a dozen plant species, it has been determined that the cell division rate on the meristem periphery exceeds that in the centre by a significant factor: from 1.5 times, in *Arabidopsis*, to 7.8 times, in *Oryza* (Laufs *et al.*, 1998b; Lyndon, 1998).

Although the central and peripheral zones are histologically separable and have different proliferative capacities, it is not clear precisely what proportion of the meristem corresponds to each region or whether a sharp boundary exists between them. However, both the central and peripheral zones do overlap the tunica and corpus, while the underlying rib zone does not overlap the tunica.

2.2.2 *Determination of meristem cell fates*

Although highly regular patterns of cell division are detected in the vegetative meristems of most plants, clonal analyses have shown that the fate of a meristem cell is determined by its position rather than its lineage (Stewart, 1978). Studies of genetic mosaics indicate that there is no fixed pattern of cell lineage, except for the general preservation of the clonal layers (Poethig *et al.*, 1986; Furner and Pumfrey, 1992; Irish and Sussex, 1992). Indeed, occasional violations of clonal restriction boundaries have been detected in chimeric analyses, without apparent consequence for the organization of the plant (Tilney-Bassett, 1986). Thus, cell commitment does not occur until relatively late in development. The undifferentiated stem cell population is considered to correspond to those cells positioned in the most apical, central region of the shoot apical meristem, whose measured divisions push their progeny cells into more peripheral and internal regions of the meristem apex (Esau, 1965). D-type cyclins, which regulate the transition from the G1 to the S phase of the cell cycle, are transcribed at high levels throughout the vegetative meristems of *Arabidopsis* and snapdragon, *Antirrhinum majus* (Riou-Khamlichi *et al.*, 1999). In fact, all cell cycle regulators investigated so far are highly expressed in the meristem and young organ primordia, reflecting the high proliferative activity of these cells (Vernoux *et al.*, 2000a). The undifferentiated apical stem cells themselves appear to be transient residents of the meristem apex, rather than acting as permanent initials (Howell, 1998). Evidence from clonal analysis suggests that the number and position of apical initial cells in cranberry, spruce and magnolia meristems changes over time (Derman, 1945; Zagorska-Marek and Turzanska, 2000), indicating that there are no permanent stem cells. Observation of mutational chimeras, in which one (or more) apical initial cell(s) was marked with a mutant sector, revealed that marked cells could be lost from the initial cell group, and that the frequency of loss in some plant species was high enough to indicate that initial cells have only a stochastic probability of persisting at the apex of the meristem (Ruth *et al.*, 1985).

Organogenesis occurs on the flanks of the meristem from small groups of cells in the peripheral zone, while the pith of the stem is derived from cells in the rib zone (Esau, 1977; Steeves and Sussex, 1989; Lyndon, 1990). The peripheral zone and the rib zone, therefore, represent transitional regions of the meristem in which undifferentiated cells derived from the central zone develop toward a more specified state in which they can become incorporated into lateral organs

or mature stem tissue. Observation of sectorized leaves, induced by irradiation of the *Arabidopsis* embryonic shoot apical meristem during probability mapping, reveals that sectors induced on later-arising leaves have a greater probability of populating two or more nodes than those induced on earlier-arising leaves (Irish and Sussex, 1992). Thus, it appears that cells in the outermost region of the meristem are more restricted in their fate, indicating that cells begin to acquire a fate as they enter the flanks of the meristem. In this way, the meristem is able to maintain a continuously replenished population of stem cells while simultaneously generating more specialized tissues that form the overall architecture of the plant.

2.3 Maintenance of the meristematic state

2.3.1 Mechanisms that promote and maintain meristem activity

The main activities of the vegetative shoot meristem following seed germination are meristem cell proliferation, organ initiation, and stem formation to generate the overall architecture of the plant. Stem formation occurs in the deeper layers of the meristem, adding breadth and girth to the shoot. Organs are produced on the flanks of the meristem, while a pool of pluripotent stem cells is maintained at the apex. To function as a site of continuous organogenesis, the shoot apical meristem must maintain a constant balance between stem cell generation and organ initiation. Thus, maintenance of the meristematic state throughout vegetative development is mediated by a complex network of positive and negative factors.

Genetic studies have identified a number of genes that promote the activity of the vegetative meristem. In angiosperms, vegetative shoot meristem maintenance requires the function of genes belonging to the plant-specific Knotted-like homeobox (*knox*) family of transcription factors (Reiser *et al.*, 2000). Two classes of *knox* genes can be distinguished, based on their sequence and gene expression patterns (Kerstetter *et al.*, 1994). Class I genes fall into a distinct clade from class II genes and are characterized by meristem-specific mRNA accumulation, while class II genes are expressed more broadly (Reiser *et al.*, 2000). Class I *knox* genes have been identified in the gymnosperm, *Picea albies*, in various grasses, and in a number of dicotyledonous species, including *Arabidopsis*, snapdragon, tomato, tobacco, soybean and apple (Reiser *et al.*, 2000). Three *knox* genes have also been cloned from the fern, *Ceratopteris*, two of which are expressed in the sporophytic shoot apex (for references see Reiser *et al.*, 2000). *knox* genes, therefore, predate the appearance of flowering plants and possibly even vascular plants, perhaps contributing to the emergence of a multiplanar, proliferative shoot meristem that occurred during the divergence of modern bryophyte and vascular plant lineages (Graham *et al.*, 2000).

The *knox* genes themselves belong to a larger superclass, the Three Amino acid Loop Extension (*TALE*) family (Burglin, 1997). *TALE* proteins are characterized by the presence of a conserved homeodomain that acts as a sequence-specific DNA binding domain, as well as two motifs termed the ELK and MIENOX domains that may be involved in protein-protein interactions (Burglin, 1997, 1998). *TALE* genes are found in animals and fungi as well as in plants, indicating a common ancestral origin (Bharanthan *et al.*, 1997; Burglin, 1997). Homeodomain transcription factors in animals are critical for pattern formation, differentiation and boundary specification during development (Carroll, 1995; Hobert and Westphal, 2000), and their duplication and diversification from ancient gene clusters is considered to have made a significant contribution to the evolution of form in metazoans (Holland *et al.*, 1994; Averof *et al.*, 1996). *knox* genes are distributed throughout the genomes of plants (Kerstetter *et al.*, 1994), but gene duplication coupled with changes in expression domains is also believed to have contributed to the diversity of *knox* gene function in plants (Reiser *et al.*, 2000).

The founding member of the *knox* homeodomain family is the maize *KNOTTED1* (*KN1*) gene (Vollbrecht *et al.*, 1991). The first indication of a role for *KN1* in cell fate specification came from the analysis of dominant mutations that caused the formation of outgrowths or 'knots' on leaves (Bryan and Sass, 1941; Freeling and Hake, 1985), a phenotype which correlated with ectopic expression of *KN1* mRNA and protein in vascular bundles of developing mutant leaves (Smith *et al.*, 1992). In wild-type plants, *KN1* is not detected in differentiated tissues, such as leaves, but instead is expressed in the subepidermal cells of vegetative and floral meristems (Smith *et al.*, 1992; Jackson *et al.*, 1994). The onset of *KN1* transcription during embryogenesis coincides with the first histological features that characterize maize shoot apical meristem formation (Smith *et al.*, 1995). After germination, *KN1* is expressed continuously throughout the shoot meristem, but is not detected in determinate lateral organ primordia (figure 2.3) (Jackson *et al.*, 1994). Overexpression of *KN1* in tobacco causes leaf lobing and the production of ectopic shoots on the leaf surface (Sinha *et al.*, 1993). The expression analysis of *KN1* in wild-type and dominant *Kn1* mutant plants, together with the gain-of-function studies, indicates that *KN1* functions to maintain cells in an undifferentiated state during development.

Loss of function mutations in *KN1* cause defects in shoot meristem maintenance, resulting in phenotypes that have been interpreted as a failure to repress the differentiation of cells within the shoot meristem itself (Kerstetter *et al.*, 1997). The most severe phenotype observed is a limited shoot phenotype, in which seedlings arrest after coleoptile formation (Vollbrecht *et al.*, 2000). This phenotype reveals an early requirement for *KN1* to maintain maize shoot apical meristem function. The penetrance of the limited shoot phenotype is dependent upon inbred background, and correlates with increased meristem height among permissive versus restrictive inbred lines. Based on these data,

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Figure 2.3 *knotted1* (*kn1*) mRNA expression in the maize shoot apical meristem. The central dome is the meristem, which is flanked by the P1 and P2 leaves (1 and 2). The patch of cells not expressing *kn1* on either side of the meristem corresponds to the predicted position of the disc of insertion of the P0 leaf (arrow) (internal scale bar = 50 μ m). (Reprinted from Jackson *et al.*, 1994. *Development*, **120** 405-413, with permission from the Company of Biologists Ltd.)

a model has been proposed for the effect of *KN1* on shoot meristem size and activity (Vollbrecht *et al.*, 2000). The proposal is that there is a size threshold in the embryo below which the meristem cannot maintain function. Meristem size in restrictive inbred lines is near the threshold, such that loss of *KN1* activity decreases the size sufficiently to push them below the critical limit. Due to the activity of one or more modifying loci, permissive inbred backgrounds have taller meristems, such that loss of *KN1* activity reduces meristem size but not below the threshold. A few other class I *knox* genes have also been characterized in maize (Kerstetter *et al.*, 1994), including *knox3*, *knox4* and *rough sheath1* (*rs1*), and these display meristem expression patterns that overlap with *KN1* (Jackson *et al.*, 1994; Kerstetter *et al.*, 1994). These data suggest that *knox* genes may provide partially redundant shoot meristem maintenance functions in maize, and may be candidates for modifiers of meristem size. The contributions of the various genes to shoot meristem maintenance may differ between different inbred backgrounds, leading to slight variations in meristem size in wild-type plants that sensitize inbreds with shorter meristems to the absence of *KN1*.

Despite the fact that *KN1* encodes a homeodomain transcription factor and, therefore, might be expected to act in a cell autonomous fashion to maintain meristem cells in an undifferentiated state, several observations indicate that

this may not be the case. Firstly, clonal analysis revealed that the formation of ectopic leaf knots requires *KN1* only in a single cell layer, although alterations in cell division and differentiation occur in all cell layers (Gelinas *et al.*, 1969; Freeling and Hake, 1985; Sinha and Hake, 1990). Secondly, *KN1* protein but not *KN1* mRNA is detected in the tunica layer of maize meristems (Jackson *et al.*, 1994), demonstrating that the protein moves between cells. Thirdly, *KN1* mRNA and protein have been reported to traffic between adjacent meristem cells via plasmodesmata (Lucas *et al.*, 1995). Non-cell autonomous function in leaves has been reported for the *Kn1*-related dominant mutation, *Gnarley1* (Foster *et al.*, 1999), but its significance is as yet unknown.

Class I *knox* genes have also been identified in other monocotyledonous plants, including barley, wheat and rice. The *HvKnox3* gene is proposed to be the barley ortholog of *KN1*, and is expressed in the meristem but not in organ primordia (Muller *et al.*, 1995). A dominant mutant of barley called *Hooded*, that induces floral meristem formation on the awn, is caused by a duplication of *HvKnox3* intron sequences, leading to ectopic expression of *HvKnox3* in the lemma of *Hooded* florets (Muller *et al.*, 1995). Thus, *HvKnox3* appears to be sufficient to maintain cells in an undifferentiated state in barley in an analogous fashion to *kn1* in maize, a conclusion supported by a report that *kn1* overexpression in barley phenocopies the *Hooded* phenotype (Williams-Carrier *et al.*, 1997). Similarly, the wheat meristem-specific class I *knox* gene, *wknx1*, a putative ortholog of *HvKnox3*, is ectopically expressed in the lemma of wheat *Hooded* mutants (Takumi *et al.*, 2000). More than six class I *knox* genes have been identified in rice (Matsuoka *et al.*, 1993; Postma-Haarsma, 1999; Sentoku *et al.*, 1999). All are transcribed during embryogenesis in the presumptive shoot apex, but after germination their expression patterns diverge (Sentoku *et al.*, 1999). While the transcripts of some rice *knox* genes are lost from the meristem after germination, the expression of *OSH1* and *OSH43* is maintained in the vegetative shoot apex. Thus these genes, like *kn1*, may be involved in maintaining meristem cells in an indeterminate state. Loss of function mutations have not been identified in either *OSH1* or *OSH43*, suggesting that they may be functionally redundant. Four *SHOOTLESS* (*SHL*) loci have been identified in rice that are required for shoot apical meristem initiation, with two of these regulating the size of the *OSH1* embryonic expression domain (Satoh *et al.*, 1999). These data reveal that the *SHL* loci are indispensable for rice shoot meristem formation and may also be involved in shoot meristem maintenance after germination.

In seedlings of *Arabidopsis* homozygous for severe recessive loss-of-function, mutations in the class I *knox* gene, *SHOOTMERISTEMLESS* (*STM*), develop normal hypocotyls, cotyledons and roots, but completely lack an embryonic shoot apical meristem and fail to produce any lateral organs (figure 2.4) (Barton and Poethig, 1993). Sections through *stm* apices show closely juxtaposed cotyledons with no trace of densely staining cytoplasmic cells between

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Figure 2.4 Scanning electron micrograph of A: an approximately 1-week-old wild-type seedling and B: a homozygous *shootmeristemless-1* (*stm-1*) *Arabidopsis* seedling. Wild-type seedlings have a functional shoot apical meristem (SAM) as determined by the presence of two leaves at the bases of the cotyledons. Seedlings homozygous for the strong *stm-1* allele do not develop a SAM (internal scale bar = 100 μ m). (Reprinted with permission from Long *et al.*, 1996. *Nature*, **379** 66-69.)

them. Instead, the cells positioned where the cotyledons meet contain storage granules characteristic of differentiated cells (Barton and Poethig, 1993). Thus, *STM* is required to establish the *Arabidopsis* shoot apical meristem but not the cotyledons, hypocotyl or roots. Plants of *Arabidopsis* carrying weaker *stm* alleles also lack an embryonic shoot apical meristem, but quickly form new shoot meristems after germination that produce a few rosette leaves before terminating (Clark *et al.*, 1996). Adventitious meristems are then generated in the axils of these organs, some of which produce limited inflorescence meristems that generate a few floral meristems before prematurely terminating. The floral meristems formed by *stm-2* mutant plants also terminate prematurely, producing reduced numbers of floral organs, particularly in the inner whorls (Clark *et al.*,

1996). These observations indicate that *STM* is also required to maintain shoot meristem activity during postembryonic development.

STM is first expressed in one or two cells of the late globular stage embryo, in the region that is predicted to give rise to the shoot apical meristem, and has a dynamic expression pattern during embryogenesis (Long *et al.*, 1996; for further discussion of the role of *STM* in embryogenesis, refer to chapter 3 of this volume). *STM* appears to function very early in embryogenesis, as it is required for expression of the *UNUSUAL FLORAL ORGANS (UFO)* gene at the early heart stage (Long and Barton, 1998). After germination, it is expressed in all of the cells of the vegetative meristem, but is excluded from developing leaf primordia. *STM* is associated with undifferentiated stem cells and is a marker for meristem activity. Every reported appearance of ectopic meristems in *Arabidopsis*, such as in the axils of *phabulosa-dominant1 (phab-1d)* leaves, is accompanied by *STM* expression (McConnell and Barton, 1998). Several other class I *knox* genes have also been identified in *Arabidopsis*, but only one of them, *KNAT1*, is expressed in the shoot apical meristem and downregulated in incipient organ primordia in a pattern similar to *STM* (Lincoln *et al.*, 1994). While no *KNAT1* loss of function mutations have been described, transgenic *Arabidopsis* overexpressing *KNAT1* can form ectopic meristems on adaxial leaf surfaces, indicating that *KNAT1* is also likely to promote shoot apical meristem cell activity (Chuck *et al.*, 1996). Interestingly, *STM* is not detected in the ectopic meristems produced by *KNAT1* overexpression lines until after they establish characteristic tunica-corporis stratification. Therefore, *STM* is not absolutely required to initiate a meristem, at least not in the context of surrounding tissues that have already undergone differentiation.

The *WUSCHEL (WUS)* gene of *Arabidopsis* is required to sustain meristematic cells after they are established (Laux *et al.*, 1996). *wus* mutants fail to organize a functional shoot apical meristem in the embryo (Schoof *et al.*, 2000). The apical meristematic cells appear to be incorrectly specified and fail to act in a coordinated fashion, undergoing differentiation without producing organ primordia (Laux *et al.*, 1996). After germination, adventitious meristems are sporadically generated at multiple foci, but these meristems are transient and form only a few organs before terminating prematurely in aberrant flat structures. Unlike wild-type meristems, which initiate organs from the flanks of the shoot apex, *wus* mutant plants initiate lateral organ primordia randomly across the entire apex. Reiterative generation and premature termination of *wus* meristems eventually produces bushy plants with multiple rosettes. Some of the *wus* meristems enter the reproductive phase and form abnormal inflorescences. These inflorescences often develop aerial rosettes of leaves, and terminate prematurely after producing a reduced number of flowers that lack inner whorl organs. These phenotypes reveal that *WUS* activity is necessary to maintain a pool of undifferentiated shoot apical stem cells (Laux *et al.*, 1996).

WUS encodes a novel subtype of the homeodomain protein family, distantly related to *STM*, that is localized to the nucleus and is predicted to function as a transcription factor (Mayer *et al.*, 1998). *WUS* transcripts are initiated prior to the appearance of *STM*, and become gradually confined to the subepidermal cells in the center of the embryonic shoot apex. After germination, the *WUS* expression domain consists of a small population of cells in the interior of the vegetative meristem, within or beneath the L3 layer (Mayer *et al.*, 1998). *WUS* and *STM* are activated independently of one another, but *STM* expression is eventually lost in *wus* mutant seedlings and vice versa. Strong *stm* alleles are epistatic to *wus* alleles, indicating that *STM* probably establishes the stem cell population upon which *WUS* acts, but mutations in *wus* enhance intermediate and weak *stm* phenotypes (Endrizzi *et al.*, 1996). Thus, the reduced meristematic activity displayed by weaker *stm* alleles requires *WUS* function, suggesting that *WUS* also acts downstream of *STM*.

The *ZWILLE/PINHEAD* (*ZLL/PNH*) and *ARGONAUTE1* (*AGO1*) genes of *Arabidopsis* are also required to maintain stem cells in the vegetative shoot apex in an undifferentiated state (Jurgens *et al.*, 1994; McConnell and Barton, 1995; Endrizzi *et al.*, 1996; Lynn *et al.*, 1999). *zll/pnh* mutants form defective shoot meristems that are reduced in size and terminate shortly after germination in a solitary, central organ. *zll/pnh* seedlings later generate adventitious meristems at the base of the cotyledons that resemble those produced by the wild-type. Ultimately, *zll/pnh* plants form inflorescences bearing slightly defective but fertile flowers, indicating that the main requirement for *ZLL/PNH* function is during embryogenesis. *ago1* mutations primarily affect leaf development (Bohmert *et al.*, 1998), but in some alleles the shoot apical meristem is occasionally replaced by a single terminal structure (Lynn *et al.*, 1999). *ago1 zll/pnh* double mutants are severely defective in vegetative shoot meristem function, and fail to express *STM* (Lynn *et al.*, 1999). Thus, *ZLL/PNH* and *AGO1* have partially redundant roles during vegetative development to promote shoot meristem activity.

Both *ZLL/PNH* and *AGO1* encode members of a novel family found in multicellular eukaryotes (Moussian *et al.*, 1998; Lynn *et al.*, 1999). This family also includes the rabbit translation initiation factor, eIF2C (Zou *et al.*, 1998), and the *piwi* gene, which is required for stem cell self-renewal in *Drosophila* and *Caenorhabditis elegans* (Cox *et al.*, 1998). Thus, the function of this gene family in the maintenance of undifferentiated cells may have ancient origins in the common ancestor of multicellular organisms. Interestingly, other members of this family are involved in RNA interference and transcription silencing in animals (Tabara *et al.*, 1999), and *AGO1* has been shown to play a role in post-transcriptional gene silencing and transgene methylation (Fagard *et al.*, 2000). One possibility for this dual function is that *ZLL/PNH* and *AGO1* may exert their effects on meristem cell activity through gene silencing mechanisms. A more likely scenario is that post-transcriptional gene silencing and meristem

maintenance share common elements or pathways. Given the resemblance between these genes and *eIF2C*, it seems reasonable that *ZLL/PNH* and *AGO1* may function in large protein complexes, components of which are used in multiple cellular processes.

Transcription of *ZLL/PNH* and *AGO1* begins early in embryogenesis (Bohmert *et al.*, 1998; Moussian *et al.*, 1998; Lynn *et al.*, 1999), but during the vegetative phase their expression patterns diverge. *AGO1* transcripts are evenly distributed throughout seedlings, while high levels of *ZLL/PNH* mRNA are detected in the vascular strands and lower levels are present in the shoot apical meristem and the adaxial domain of developing leaves (Moussian *et al.*, 1998; Lynn *et al.*, 1999). *ZLL/PNH* function is necessary to maintain high levels of *STM* expression late in embryonic shoot apical meristem development (Moussian *et al.*, 1998). Thus, transient high level *ZLL/PNH* expression in the embryonic apex may prevent *STM* downregulation and consequent meristem differentiation, possibly through a cell autonomous mechanism of silencing or limiting the range of negative regulators of *STM*. Alternatively, *ZLL/PNH* may play a role in signaling from the somatic provascular tissue to the overlying meristem cell population to maintain *STM* expression and shoot meristem activity (Moussian *et al.*, 1998; Lynn *et al.*, 1999). A similar mechanism has been proposed for the *piwi* gene of *Drosophila*, which functions to maintain germ cells in an undifferentiated state, and which is not expressed in the germ cells themselves but in the underlying somatic tissue.

The tomato *DEFECTIVE EMBRYO AND MERISTEMS (DEM)* gene is required for proper organization and development of both the shoot and root apex (Keddie *et al.*, 1998). *DEM* encodes a novel protein, with homologs in *Arabidopsis* and yeast, that is expressed in a complicated pattern during development. Reminiscent of *ZLL/PNH*, *DEM* is expressed at high levels in shoot apices and also in the vasculature and on the adaxial side of developing leaves. *DEM* mRNA is also present in root tips and other tissues in which organized cell divisions occur, but as it is not detected in callus, it appears that *DEM* is not a component of the cell cycle machinery or a general regulator of cell division (Keddie *et al.*, 1998). *DEM* may thus represent a new class of regulatory genes that affect a basic aspect of meristem function that is common to both shoots and roots.

2.3.2 *Non-autonomous signaling mechanisms that regulate the size and organization of meristematic tissues*

Shoot-apical-meristem-promoting activities, such as those provided by *STM* and *ZLL/PNH*, are counterbalanced by non-autonomous signaling pathways that limit stem cell activity. *fasciated* mutants of the tomato, *Lycopersicon esculentum*, have enlarged vegetative and floral meristems that grow as rings or straps instead of points (Merton and Burdick, 1954), indicating that a control

mechanism designed to restrict meristem growth activity has failed. The creation of chimeras between wild-type and *fasciated* plants revealed that the genotype of the L3 cell layer alone determined whether the meristem was normal or enlarged, indicating that the L1 and L2 cell layers respond to signals sent from the L3 (Szymkowiak and Sussex, 1992). The *fasciata1* (*fas1*) and *fasciata2* (*fas2*) mutations of *Arabidopsis* cause a similar meristem fasciation phenotype, disrupting meristem growth control and cellular organization in both the shoot and root apical meristem (Leyser and Furner, 1992; Kaya *et al.*, 2000).

Loss-of-function mutations at the *Arabidopsis* *CLAVATA* (*CLV1-3*) loci cause a specific increase in the size of the shoot apical meristem beginning during embryogenesis. Excess stem cells continue to accumulate throughout development, leading to stem fasciation and the production of flowers with extra floral organs (figure 2.5) (Leyser and Furner, 1992; Clark *et al.*, 1993, 1995; Kayes and Clark, 1998). From a wealth of genetic, molecular and biochemical evidence it has been concluded that *CLV1*, *CLV2* and *CLV3* act in the same

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Figure 2.5 Apical meristems of wild-type and *clavata1* (*clv1*) plants at day 8 of *Arabidopsis* vegetative development. A: Landsberg; B: *clv1-5*; C: *clv1-1*; and D: *clv1-4* plants were examined by scanning electron microscopy. The arrows indicate leaf primordia. All plants carried the *erecta* mutation (internal scale bar = 10 μ m). (Reprinted from Clark *et al.*, 1993. *Development*, **119** 397-418, with permission from the Company of Biologists Ltd.)

pathway to regulate shoot apical meristem cell fate. Double mutants carrying weaker *clv1* and *clv3* alleles display severe *clv* mutant phenotypes, while those carrying strong *clv1* and *clv3* alleles show mutual epistasis (Clark *et al.*, 1995). Doubly heterozygous *clv1/+ clv3/+* mutants display an intermediate *clv* phenotype, indicative of non-allelic non-complementation and the sensitivity of each protein to a reduction in the level of the other. *CLV2* acts in the same meristem growth control pathway as *CLV1* and *CLV3*, but also functions more widely during development (Kayes and Clark, 1998). *clv1* and *clv3* alleles dominantly suppress *stm* mutant phenotypes and vice versa, indicating that *CLV1/CLV3* and *STM* act antagonistically to regulate shoot apical meristem function (Clark *et al.*, 1996).

The three *CLV* genes form critical components of a signal transduction pathway that communicates stem cell fate information between neighboring groups of shoot apical meristem cells. The *CLV1* gene encodes a protein containing extracellular leucine-rich repeats (LRRs), a transmembrane domain and an intracellular serine/threonine kinase domain (Clark *et al.*, 1997). The *CLV2* gene encodes an LRR receptor-like protein that consists of extracellular LRRs, a transmembrane domain and a short cytoplasmic tail (Jeong *et al.*, 1999). *CLV1* and *CLV2* are members of large families found both in plants and animals. There are 174 putative LRR receptor kinases encoded by the *Arabidopsis* genome, and at least 30 receptor-like proteins that resemble *CLV2* (Initiative, 2000). Many plant LRR receptor kinases are involved in cell signaling (Becraft, 1998), and both in plants and animals LRRs are a common motif of protein binding domains (Buchanan and Gay, 1996), suggesting that ligand binding by the *CLV1* and *CLV2* receptors occurs via their extracellular LRR domains. The *CLV3* gene encodes a protein of lower molecular mass that is predicted to be extracellular, and with no significant homology to other known plant or animal proteins (Fletcher *et al.*, 1999). Mosaic analysis of periclinal chimeras, generated using an unstable *clv3* allele, has revealed that *CLV3* can act in a cell non-autonomous fashion (Fletcher *et al.*, 1999), as is characteristic of signaling molecules.

Biochemical evidence strongly indicates that *CLV1*, 2 and 3 are bound together in a signaling complex. The *CLV1* protein is present in two complexes *in vivo*, a 180 kDa complex and a larger 450 kDa heteromeric complex (Trotochaud *et al.*, 1999). The 450 kDa complex does not form in *clv1-10* mutants that have an inactive kinase domain, indicating that this form represents the active complex and that the 185 kDa complex is an inactive complex. Moreover, the 450 kDa complex does not form in *clv2* or *clv3* mutants, so all three elements must be present for proper association of the active signaling complex. Experiments have shown that the *CLV3* protein binds specifically to the active form of *CLV1*, and that *CLV3* associates with the *CLV1/CLV2* receptor complex at the plasma membrane in yeast (Trotochaud *et al.*, 2000). These data convincingly demonstrate that *CLV3* is the ligand for the *CLV1* receptor complex, a complex which is likely to include *CLV2*.

The CLV1 active complex also contains several associated proteins that interact with CLV1 on the cytoplasmic side of the plasma membrane. The CLV1 kinase domain interacts with a kinase-associated protein phosphatase (KAPP), which is expressed in a domain encompassing the *CLV1* expression domain (Stone *et al.*, 1994, 1998; Williams *et al.*, 1997). The KAPP kinase interaction domain contains a phosphoserine/phosphothreonine binding forkhead-associated (FHA) domain (Li *et al.*, 1999), suggesting that KAPP may bind phosphoserine residues in the CLV1 kinase domain. Based on the analysis of KAPP overexpression lines that phenocopy weak *clv* mutants, the KAPP phosphatase is proposed to dephosphorylate CLV1 and act as a negative regulator of the pathway (Williams *et al.*, 1997).

The active form of the CLV complex also includes at least one member of a plant Rho/Rac GTPase-related protein subfamily called Rop (Li *et al.*, 1998). *Arabidopsis* contains at least ten Rop family members, many of which are expressed in shoots (Li *et al.*, 1998), but it is not yet clear which Rop or Rops participate in CLV signaling. Rho/Rac GTPases are members of the Ras GTPase superfamily, and are conserved signaling proteins that mediate many receptor tyrosine kinase signaling events in animals and fungi to control key cellular processes, such as actin cytoskeletal reorganization, cell polarity and polarized cell growth (Chant and Stowers, 1995; Nagata and Hall, 1996). Ras GTPases are not present in the *Arabidopsis* genome (Initiative, 2000) and have yet to be found in plants. Thus, Rho/Rac GTPases, such as the Rop proteins, may functionally substitute for Ras GTPases in plant signal transduction. Based on analogous roles for Ras GTPases in animals, Rop GTPases may serve in the vegetative meristem to activate a mitogen-activated protein kinase (MAPK)-like cascade in response to CLV1 kinase activation.

The *CLV3* and *CLV1* mRNAs are expressed initially in the presumptive embryonic shoot apical meristem about midway through embryogenesis. After germination, *CLV3* mRNA accumulates in a small group of cells at the very apex of the vegetative meristem, predominantly in the L1 and L2 tunica cells of the region corresponding to the central zone (Fletcher *et al.*, 1999). *CLV1* mRNA is expressed in the deeper regions of the central part of the vegetative meristem, mainly in the L3 corpus layers. *CLV1* mRNA is not detected in the L1 cell layer and, at least in the shoot apical meristem, is also absent from the L2 layer (Clark *et al.*, 1997). Thus, the *CLV3* expression domain overlies the *CLV1* expression domain, indicating that these two populations of meristem cells communicate with one another via the CLV signaling pathway. The *CLV2* gene is also expressed in shoots and developing flowers, as well as more broadly throughout the plant (Jeong *et al.*, 1999).

In *clv1*, 2 or 3 mutants, the expression domains of both *CLV1* and *CLV3* enlarge coordinately (Fletcher *et al.*, 1999; Jeong *et al.*, 1999). These data have been interpreted to mean that the coordinated expansion of the CLV expression domains is controlled by a positive, stem-cell-promoting pathway, which in turn

is negatively regulated by the stem-cell-restricting *CLV* pathway. These two pathways were uncoupled by overexpressing *CLV3* in transgenic *Arabidopsis* plants (Brand *et al.*, 2000). Analysis of the transgenic plants indicated that stem cells are not correctly maintained when *CLV3* is constitutively expressed at high levels. Furthermore, the abundance of the *CLV3* ligand was shown to be the critical factor that determines the size of the stem cell pool and, consequently, the number of lateral organs that can be produced by the meristem. The transgenic *CLV3* overexpressing lines were also used to demonstrate that *CLV3* signaling requires functional *CLV1* and *CLV2*, and that the loss of stem cells observed in the transgenic *CLV3* plants is the result of enhanced *CLV3* signaling through the *CLV1/CLV2* receptor complex.

The *WUS* gene has been identified as a critical target of *CLV* meristem signal transduction. *wus* mutations are epistatic to *clv1* and *clv3* mutations (Schoof *et al.*, 2000), suggesting that *WUS* functions downstream of the *CLV* complex to promote stem cell activity, perhaps acting at the transcriptional level, and that its activity is downregulated by *CLV* signaling. In wild-type plants, *WUS* expression is restricted to a small group of L3 meristem cells, beneath the *CLV3* expression domain and overlapping the *CLV1* domain (Mayer *et al.*, 1998). Maintenance of the *WUS* expression domain requires the activity of FAS1 and FAS2, components of the chromatin assembly factor-1 from *Arabidopsis* (Kaya *et al.*, 2000) that are likely to promote stable *WUS* gene transcription by facilitating chromatin assembly after DNA replication. In *clv3* mutant meristems, the *WUS* expression domain expands laterally and also upward into the subepidermal layer (Brand *et al.*, 2000; Schoof *et al.*, 2000). Conversely, *WUS* mRNA is not detected in the transgenic *CLV3* overexpressing plants, which phenocopy the *wus* loss-of-function mutant phenotype (Brand *et al.*, 2000). Thus, signaling through the *CLV* pathway leads to restriction of the *WUS* expression domain and negative regulation of stem cell activity. Mis-expression of *WUS* on the meristem periphery is sufficient to induce *CLV3* transcription and transform all lateral organ primordia into undifferentiated meristematic cells (Schoof *et al.*, 2000). Thus, *WUS* is sufficient to specify stem cell fate, and is one component of a stem-cell-promoting pathway that controls the stem-cell-restricting pathway by regulating the size of the *CLV3* expression domain.

Thus, in wild-type plants of *Arabidopsis*, the *CLV3* signal originates from the stem cells at the meristem apex and is sensed by the underlying L3 cells. The *CLV3* ligand is probably bound by the *CLV1/CLV2* receptor complex through their extracellular LRRs, activating the complex and leading to autophosphorylation of the *CLV1* kinase domain. Signaling through the *CLV* pathway restricts *WUS* activity by limiting its expression to a small group of cells in deeper layers of the meristem (figure 2.6). Constitutive signaling through *CLV3* enhances this negative pathway, causing *WUS* downregulation and complete loss of stem cells. When the negative pathway is disrupted in *clv* mutants, the *WUS* expression

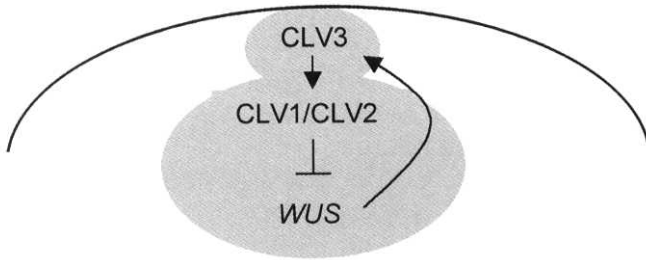


Figure 2.6 A negative feedback loop regulates vegetative meristem cell fate in *Arabidopsis*. Cell-non-autonomous signaling via *WUSCHEL* (*WUS*) promotes the transcription of *CLAVATA3* (*CLV3*) and maintenance of stem cell fate in the overlying apical meristem cells. *CLV3* expression in the overlying cells activates a *CLV1/CLV2* receptor complex in the interior. Signal transduction through the *CLV* complex leads to the downregulation of *WUS* transcription, limiting the *WUS* meristem-promoting activity to a narrow domain. (Reprinted from Fletcher and Meyerowitz, 2000. *Current Opinion in Plant Biology*, 3 23-30.)

domain expands laterally and upward as excess stem cells accumulate. Activity of the positive pathway mediated by *WUS* promotes *CLV3* expression, and maintenance of the *CLV3* expression domain. Since *WUS* mRNA is not detected in the L1 or L2 layers of wild-type meristems, an inductive signal mediated by *WUS* probably maintains meristem cell activity in the superficial cell layers. Thus stem cell activity in vegetative meristems of *Arabidopsis* is mediated by mutual regulation and signaling across the layers of the meristem, involving both positive and negative interactions. Such regulation provides a stable feedback system that tends towards equilibrium, achieving the delicate balance required to maintain a small pool of undifferentiated stem cells throughout the life of the plant.

2.3.3 Contribution of hormones to meristem maintenance

It has long been known that increasing the ratio of cytokinin relative to auxin induces the formation of shoots from callus (Skoog and Miller, 1957), implicating cytokinin in shoot apical meristem induction. Adventitious shoot formation on leaves has also been observed in plants overexpressing the isopentenyl transferase gene of *Agrobacterium tumefaciens*, which is involved in cytokinin synthesis (Estruch *et al.*, 1991; Li *et al.*, 1992). More recent work is beginning to shed light on possible molecular mechanisms for cytokinin function in the shoot meristem. The mRNA levels of two D-cyclin genes expressed in *A. majus* meristems increase in response to cytokinin application (Gaudin *et al.*, 2000), indicating that cytokinin may modulate the entry of meristem cells into the cell cycle. D3-cyclin overexpression mimics the effects of cytokinin on the cell cycle (Riou-Khamlichi *et al.*, 1999), suggesting that cytokinin may increase the rate at which meristem cells progress through the cell cycle by promoting D3-cyclin

activity. Further discussion on the regulation of the cell cycle in the meristem is included in chapter 8 of this volume.

Transgenic tobacco plants that overexpress maize *kn1* or its *Arabidopsis* homolog, *KNAT1*, display phenotypes that resemble those of cytokinin-overproducing tobacco plants (Hewelt *et al.*, 1994), such as reduced apical dominance and production of ectopic shoots (Sinha *et al.*, 1993; Chuck *et al.*, 1996). *Arabidopsis amp1* mutants that produce higher than normal levels of cytokinins have pleiotrophic phenotypes that include enlarged shoot apical meristems, increased branching and accelerated leaf initiation (Chaudhury *et al.*, 1993; Chin-Atkins *et al.*, 1996). A D3-cyclin is overexpressed in the *amp1* mutants, confirming the link between cytokinin and cell cycle regulation in the meristem. Transgenic seedlings of *Arabidopsis* that overproduce endogenous cytokinin have phenotypes similar to those of the *amp1* mutants, including increased biomass production and slightly reduced apical dominance (Rapp *et al.*, 1999). Shoot apical meristem size is not altered in the cytokinin overproducing transgenic plants, but the steady-state mRNA levels of both *STM* and *KNAT1* are enhanced. Although alternative hypotheses cannot be ruled out, the data are consistent with a model in which cytokinin acts upstream of *STM* and *KNAT1*, playing a natural role in shoot apical meristem maintenance.

2.4 Organogenesis in the vegetative meristem

2.4.1 Regulation of organ initiation

A major function of the shoot apical meristem during vegetative development is the generation of the various morphological forms that contribute to the overall architecture of the plant. Lateral structures that originate as outgrowths from the indeterminate vegetative meristem must acquire distinctive characteristics that culminate in the formation of a determinate leaf. In order for this to occur, groups of cells must be selectively set aside from the shoot apex and progressively restricted from certain cell fates. These founder cells are then permitted to proceed down a pathway or pathways that lead to their differentiation, and the differentiation of their progeny cells, into specialized tissues with limited developmental potential. Clonal analysis indicates that leaf primordium initiation occurs within a single rank of cells in the subepidermal layer (Irish and Sussex, 1992), and propagates across the layers into a somewhat variable number of founder cells (Furner and Pumfrey, 1992; Irish and Sussex, 1992). This number ranges from 5–10 cells per layer in *Arabidopsis* (Furner and Pumfrey, 1992; Irish and Sussex, 1992) to 50–100 cells per layer in maize (Poethig and Szymkowiak, 1995) and tobacco (Poethig and Sussex, 1985b). Initiation of a leaf primordium is achieved through coordinated changes in cell division rates and planes within the group of founder cells, coupled with directional cell expansion and growth away from the meristem. Organ initiation events thus consist of recruitment and

specification of founder cells on the meristem periphery, initiation of the lateral organ primordium, and the separation and outgrowth of the primordium. Organ initiation events are precise processes that are regulated both spatially, in terms of the phyllotaxy or positioning of an initiation site relative to other primordia, and temporally, in terms of the plastochron or time interval between successive organ initiation events.

Three *SHOOT ORGANIZATION (SHO)* loci regulate leaf founder cell recruitment in the early vegetative meristem of *Oryza sativa*. *sho* mutants have a defective shoot meristem organization, generating broader and flatter vegetative apices than wild-type plants that produce supernumerary threadlike leaves from randomly distributed initiation sites (Tamura *et al.*, 1992; Itoh *et al.*, 2000). The accelerated and random leaf production is associated with increased cell division rates across the *sho* meristem, and with a reduction in the expression domain of the class I *knox* gene, *OSH1* (Itoh *et al.*, 2000). These phenotypes indicate that *sho* mutant meristems contain excess cells in the peripheral region relative to the central region, and that these cells are rapidly incorporated into random organ primordia initiation sites. The *SHO* loci are therefore proposed to control the rate at which cells make the transition from the central zone into the peripheral zone of the meristem, maintaining proper meristem organization and leaf primordia initiation patterning by limiting the number of founder cells available for recruitment into organ primordia. Interestingly, *SHO* gene activity is not necessary during the late vegetative phase of rice development, indicating that meristem disorganization does not affect leaf production at this stage or that another, phase-specific gene is able to compensate for the lack of *SHO* function.

The *MGOUN1 (MGO1)* and *MGOUN2 (MGO2)* genes of *Arabidopsis* are also required for the proper partitioning of peripheral meristem cells into lateral organ primordia. *mgo1* and *mgo2* mutants have fasciated meristems with an enlarged peripheral zone, but they produce fewer lateral organs than wild-type plants (Laufs *et al.*, 1998a). Thus, *mgo* mutants contain excess meristem cells, but these cells are not properly allocated into organ primordia and continue to accumulate on the meristem periphery. Unlike *sho* mutants, *mgo* mutant meristems are relatively normal in their cellular organization, indicating that the effect of *mgo* on founder cell partitioning is specific and not a by-product of a general disruption of meristem structure. The allocation of peripheral meristem cells into organ primordia therefore appears to be strictly controlled, so that the population of undifferentiated stem cells is not depleted.

STM may also be involved in regulating organogenesis on the meristem flanks. The observation that *STM* is expressed initially in the peripheral region of the embryonic shoot apical meristem, and only later in the center, has led to the proposal that *STM* plays two roles in the shoot apex (Long and Barton, 1998). One function is in the central region, to maintain stem cells in an undifferentiated state, and the other is on the periphery, to inhibit premature outgrowth of lateral organs. Partial loss-of-function mutations in *STM* randomize

the positioning of organ initiation sites and also reduce the rate at which leaves are initiated (Clark *et al.*, 1996), indicating that *STM* activity is required for proper spatial and temporal patterning during lateral organ initiation. Mutations in *STM* also affect the regulation of the *Arabidopsis* cytochrome P450 gene, *CYP78A5*, which is strongly expressed on the periphery of the vegetative meristem marking a region between the central domain and the developing organ primordia (Zondlo and Irish, 1999). In *stm* mutants, *CYP78A5* is transcribed at decreased levels uniformly across the meristem, indicating that *STM* negatively regulates *CYP78A5* in the centre of the meristem and positively regulates it on the periphery. Several other cytochrome P450 proteins from *Arabidopsis* are involved in the regulation of cell expansion and directional cell growth (Szekeres *et al.*, 1996; Kim *et al.*, 1998), which suggests the possibility that *CYP78A5* plays a role in controlling growth and organogenesis in the peripheral region of the meristem. Regulation of *CYP78A5* by *STM* may ensure that organ initiation events occur only on the flanks of the meristem, where rapid cell divisions produce populations of organ founder cells, rather than in the centre, where primordia initiation could consume the entire meristematic region.

2.4.2 Establishment of organ initiation sites

Changes in *knox* gene expression patterns represent the first detectable molecular indicator of leaf formation. The class I *knox* genes, *kn1* and *STM*, are expressed throughout the shoot apical meristem, where they function to maintain stem cells in apices of maize and *Arabidopsis*, respectively, in an undifferentiated state. Both genes are downregulated on the meristem flanks just prior to the emergence of lateral organ primordia as morphologically distinct buttresses. The number of cells that exhibit loss of *kn1* expression is similar to the number predicted from clonal analysis to found the leaf (Poethig and Szymkowiak, 1995). The timing of *kn1* downregulation in maize organ primordia also coincides with the time at which the site of leaf initiation in other angiosperms is fixed (Snow and Snow, 1933) and the determinate nature of the leaf, as evidenced by the acquisition of dorsiventrality, is established (Sussex, 1955; Snow and Snow, 1959). Thus, negative regulation of *kn1* and *STM* genes in organ founder cells on the flanks of the vegetative meristem may be a necessary prerequisite for these cells to become committed to their developmental fates, and consequently for the initiation of the coordinated changes in peripheral meristem cell behavior that generate a morphologically distinct leaf buttress (for further discussion refer to chapter 7 of this volume).

The acquisition of organ founder cell fate is associated with the expression of several genes in snapdragon, maize and *Arabidopsis* that encode putative transcription factors. Expression of the *PHANTASTICA* (*PHAN*) gene of *A. majus* is confined to lateral organ primordia and correlates with the determination of leaf

founder cells (Snow and Snow, 1933). *PHAN* encodes a putative Myb domain transcription factor necessary to elaborate the proximodistal axis during lateral organ initiation, and to generate dorsoventral asymmetry in developing leaves and petals (Waites and Hudson, 1995; Waites *et al.*, 1998). *PHAN* and its maize homolog, *rough sheath2* (*rs2*), are required for the downregulation of the *knox* genes, *AnSTM* and *kn1*, respectively, on the meristem flanks (Waites *et al.*, 1998; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999). The primary role of *PHAN* and *rs2* is therefore proposed to be to specify the identity of lateral organ cells as distinct from those in the meristem. *PHAN* also appears to maintain meristem activity in a cell non-autonomous fashion (Waites *et al.*, 1998), indicating that *PHAN* is one component of a long recognized system that transmits signals from the lateral organs back to the shoot apical meristem (Steeves and Sussex, 1989).

Analysis of the *Myb* gene, *ASYMMETRIC LEAVES1* (*AS1*), from *Arabidopsis* has revealed a possible mechanism for leaf founder cell specification via region-specific regulation of transcription factor gene expression. Like *PHAN*, *AS1* is expressed only in incipient organ primordia in a reciprocal domain to *STM* (Byrne *et al.*, 2000). In stem cells at the meristem apex, *STM* negatively regulates *AS1*, which causes phenotypes similar to those observed in *knox* gene overexpressing lines. In lateral organ founder cells, *STM* is downregulated, not by *AS1* itself (Byrne *et al.*, 2000) but potentially by another Myb-like gene that has a function similar to *PHAN*. This action lifts one check on cell differentiation in the founder cells, allowing *AS1* to be transcribed in these regions. *AS1* activity negatively regulates the expression of the meristem-specific homeobox gene, *KNAT1*, further lifting the requirement that the founder cells remain in an undifferentiated state and allowing them to proceed into pathways leading to specification as leaf cells (Byrne *et al.*, 2000). This pathway of negative interactions between *Myb* and *knox* genes functions to distinguish organ primordium cells from stem cells in the shoot apical meristem in a tightly controlled manner (figure 2.7).

Expression of the *Arabidopsis* *AINTEGUMENTA* (*ANT*) gene, which encodes a member of the AP2 family of transcription factors, is also restricted to lateral organ primordia (Elliot *et al.*, 1996). However, *ANT* and *AS1* appear to play distinct roles in organogenesis. Loss-of-function mutations in *ANT* cause ovule defects and a reduction in lateral organ number and size that correlates with decreased cell number (Elliot *et al.*, 1996; Klucher *et al.*, 1996). Conversely, gain of *ANT* function enlarges organ size by increasing cell number, without affecting organ morphology (Mizukami and Fischer, 2000). In addition, ectopic *cycD3* expression is observed in the mature leaves of *ANT* overexpressing plants, indicating that *ANT* could exert a direct effect on cell accumulation in developing organ primordia by controlling cell cycle progression. *ANT* may therefore determine the extent of lateral organ growth by sustaining cell proliferation coupled to cell growth during organogenesis (Mizukami and Fischer, 2000).

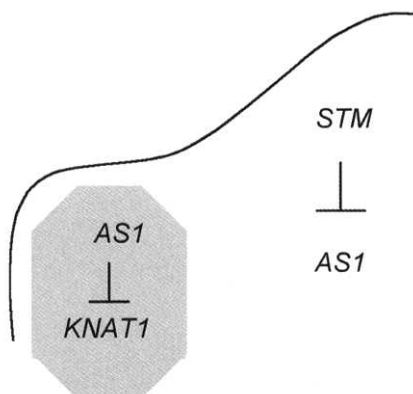


Figure 2.7 Specification of leaf founder cells in *Arabidopsis* vegetative meristems. SHOOTMERISTEMLESS (*STM*) activity negatively regulates *ASYMMETRIC LEAVES1* (*AS1*) expression in the central region of the meristem. Downregulation of *STM* in incipient organ primordia permits *AS1* expression, which in turn negatively regulates the meristem-specific homeobox gene, *KNAT1*. (Based on Byrne *et al.*, 2000. *Nature*, **408** 967-971.)

The region-specific expression of transcriptional regulatory genes, such as *PHAN*, *AS1* and *ANT*, in organ founder cell populations may in turn induce the localized expression of target genes that bring about the changes in cell division rates, planes of orientation and localized cell expansion that culminates in a morphologically distinct organ primordium. One group of putative target effector molecules are the expansins (Reinhardt *et al.*, 1998). Expansins are extracellular proteins that increase the extensibility of plant cell walls *in vitro* and are proposed to play roles in cell expansion (Keller and Cosgrove, 1995; Cho and Kende, 1997; Cosgrove, 1997). Exogenous application of purified expansin protein on the flanks of tomato vegetative apices induces local outgrowth of leaf-like primordia, indicating that exogenous expansin can induce some aspects of the endogenous primordia initiation program (Fleming *et al.*, 1997). The *LeExp18* gene is expressed at low levels throughout the tomato meristem but is upregulated at the sites of incipient organ primordium initiation (Reinhardt *et al.*, 1998). This suggests that *LeExp18* could be an immediate target of founder cell transcription factors that set in motion the early morphological events associated with leaf formation (for further discussion, refer to chapter 6 of this volume).

The earliest morphologically detectable event in the initiation of a leaf primordium is an increased frequency of periclinal divisions among tunica cells in the meristem peripheral zone (Medford *et al.*, 1994), which is closely followed by a reorientation of cell division planes in the corpus (Esau, 1965). These alterations in cell division planes may stimulate, or be stimulated by, mechanical forces. Recent data indicate the presence of a zone of compression in the sunflower capitulum, which could control the initiation of new lateral organ

primordia via the buckling of the tunica layer (Dumais and Steele, 2000). Expansins have been suggested to induce changes in the cell wall extensibility of peripheral meristem cells that could lead to such altered physical stress patterns (Fleming *et al.*, 1997), and possibly coordinate the redirection of cell division planes with localized changes in cell expansion. Cells from all layers of the meristem contribute to the formation of a lateral organ (Szymkowiak and Sussex, 1996), indicating that alterations in cell division rates and planes of orientation must be coordinated between them in order to ensure proper organogenesis. Mechanical propagation of a small compression zone across cell layers might be one method of achieving this end. Localized reorientation of cellulose microfibrils on the flanks of the meristem, perhaps driven by a corresponding shift in the orientation of cortical microtubules, also accompanies leaf initiation (Hardham *et al.*, 1980; Jesuthasan and Green, 1989). Such alterations in cellulose deposition are thought to be necessary to permit the change in cell division polarity associated with organ outgrowth (for further discussion, refer to chapter 6 of this volume).

The rate of division of cells on the meristem flank increases, although modestly, approximately one half plastochron prior to the emergence of a leaf primordium (Laufs *et al.*, 1998b; Lyndon, 1983). This increase persists until the primordium has initiated and then falls back to its original level. Another consequence of founder-cell-specific induction of transcriptional regulators may therefore be at the level of the cell cycle machinery, affecting local control of cyclins such that the meristem cells recruited into organ primordia can begin to divide more rapidly. At least one cyclin gene, *cyclin D3a* from *Antirrhinum*, is preferentially expressed on the meristem periphery (Gaudin *et al.*, 2000), implying that differences in proliferative capacity between the central and peripheral regions may be established by region-specific expression of cell cycle regulators. Interestingly, D2-type cyclin overexpression throughout transgenic tobacco plants does not cause an increase in meristem size, but instead accelerates the overall growth rate and the rate of initiation of new leaves (Cockcroft *et al.*, 2000). Therefore, the plastochron appears to be determined by the cell division rate within the shoot meristem, reflecting the requirement of the meristem to replenish the cell population on its flanks prior to the initiation of a new organ primordium (for further discussion, refer to chapter 8 of this volume).

The relative contribution of increased cell division rates, changes in the orientation of cell division planes, and localized cell expansion to the initiation of leaf primordia is a matter of some debate. Remarkably, lateral organ initiation can take place in the absence of precise reorientation of the planes of cell division, and also in the absence of an increase in the rate of cell division. Mutants of *Arabidopsis*, such as *ton1* and *fass1*, which disrupt the alignment of cell division planes such that cell divisions occur in random orientations, have functional meristems that produce recognizable lateral organs, albeit with

abnormal morphology (Traas *et al.*, 1995). Conversely, in heavily irradiated wheat seedlings where cell divisions are absent, leaf primordium outgrowth through polarized cell expansion is still detected (Foard, 1971). However, changes in localized cell expansion brought about by exogenous expansin application to meristem surfaces are not in themselves sufficient to induce the formation of morphologically normal leaves, and in any case are accompanied by cell division (Fleming *et al.*, 1997). Therefore, all three morphological events appear to be necessary for proper initiation of leaf primordia on the vegetative meristem flanks.

Finally, the patterning of lateral organ primordia initiation sites in the maize meristem occurs through the activity of the *terminal ear1 (te1)* gene. *te1* mutants display increased rates of leaf initiation, short internodes and aberrant phyllotaxy that are associated with abnormal vegetative meristem geometry (Veit *et al.*, 1998). *te1* transcripts are detected in the vegetative shoot apex in a series of semicircular bands. Leaf initiation sites are established between these bands, where *te1* mRNA levels are lowest. One model for *te1* function is that it inhibits the cells in which it is expressed from acting as organ founder cells, preventing initiation events from occurring too rapidly or too closely together and establishing the internode region between organ initiation sites. Because *te1* mutant plants have abnormal phyllotaxy and also more internodes, *te1* may regulate both the spatial and temporal patterning of leaf primordium initiation. The *te1* gene product is similar to the *Schizosaccharomyces pombe* RNA-binding protein, Mei2, which is required for commitment to meiosis (Watanabe *et al.*, 1988). The related *Arabidopsis FCA* gene regulates flowering time, indicating that the plant members of this family act in multiple developmental processes and that the similarity between them may reflect the conservation of an underlying RNA interaction mechanism (for further discussion, refer to chapter 7 of this volume).

2.4.3 Establishment of boundaries between the meristem and organ primordia

A critical event during vegetative organogenesis is the establishment of the initiating leaf primordium as a distinct entity from the meristem by the creation of a boundary between them. Several genes have been identified in monocotyledonous and dicotyledonous plants that appear to be involved in boundary formation, based on their expression patterns and mutant phenotypes. The class I *knox* genes, *rough sheath1*, *knox3* and *knox4*, are all expressed in rings encircling the vegetative meristem, but between the developing lateral organs (Jackson *et al.*, 1994; Schneeberger *et al.*, 1995). Similarly, the expression of both *Oskn3* and of *OSH15*, a putative ortholog of *knox4/rs1*, is downregulated in the vegetative meristem of rice and becomes localized at the boundaries of lateral organs (Sato *et al.*, 1998; Postma-Haarsma, 1999). These *knox* genes

may therefore play roles in defining the segmental regions, the internodes, between lateral organs. Loss-of-function mutations in *OSH15* cause dwarfing and a reduction in internode length, consistent with this hypothesis.

The *no apical meristem* (*nam*) gene product is required for formation of the embryonic shoot apical meristem in *Petunia*, and for proper positioning and separation of the cotyledons (Souer *et al.*, 1996). The *nam* meristem phenotypes are reminiscent of *stm* meristem phenotypes, but unlike *STM* and other meristem-promoting genes, *nam* is not expressed in the meristem. Instead, *nam* mRNA accumulates at the boundaries between meristems and lateral organ primordia (figure 2.8), indicating that during postembryonic development the major function of *nam* is to specify the relative position of primordia relative to the meristem. *nam* encodes a putative NAC-domain transcription factor (Souer *et al.*, 1996) and is a member of a gene family (Sablowski and Meyerowitz, 1998) found in the dicotyledonous species, *Petunia* and *Arabidopsis*. Another NAC gene, the *CUP-SHAPED COTYLEDONS2* (*CUC2*) gene of *Arabidopsis*, is also involved in organ initiation and separation and is expressed, like *nam*, in a ring around initiating lateral organ primordia (Aida *et al.*, 1997; Ishida *et al.*, 2000). Thus, members of this gene family are required to establish boundaries between shoot meristems and organ primordia, as well as to separate organ primordia from one another. Several studies have indicated that the rate of cell division and the direction of cell expansion in floral meristems differs between primordial regions and the regions between (Hernandez *et al.*, 1991;

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Figure 2.8 *no apical meristem* (*nam*) mRNA expression pattern in wild-type *Petunia* embryos and inflorescence meristems. A: Torpedo stage embryo in which *nam* expression is limited to a ring around the developing shoot apical meristem. B: Inflorescence apex—the two vertical stripes of *nam*-expressing cells are at the boundaries of the site at which bract primordia will appear; the horizontal *nam* stripe at the bottom marks the boundary of a bract formed at an earlier node. C: Inflorescence apex with two bracts—*nam* expression separates the two bracts and the floral and inflorescence meristems. (Reprinted from Souer *et al.*, 1996. *Cell*, **85** 159-170, with permission from Elsevier Science.)

Vincent *et al.*, 1995). If the same holds true in vegetative meristems, the expression of *nam* and *CUC2* in a narrow domain encircling the lateral organ primordia may enable them to determine the boundaries between organs and the meristem by inhibiting the growth of cells at the interface.

Meristem-organ boundary formation in *Arabidopsis* also appears to be affected by localized auxin distribution controlled by the *PIN-FORMED1* (*PIN1*) gene. Loss of *PIN* function severely reduces polar auxin transport and curtails organ initiation, causing a reduction in leaf number and the formation of a naked inflorescence meristem (Okada *et al.*, 1991; Bennett *et al.*, 1995). *PIN1* encodes a putative auxin efflux carrier protein that is expressed in vascular tissue and at low levels throughout the shoot apical meristem (Galweiler *et al.*, 1998). *PIN1* mRNA expression is upregulated on the flanks of the meristem just prior to the emergence of the organ primordia (Vernoux *et al.*, 2000b), indicating a possible role for *PIN1* in the early stages of organ initiation. The cells on the periphery of *pin1* mutant meristems express *ANT*, indicating that they have acquired some characteristics of lateral organs, but fail to progress to later stages of primordium outgrowth (Vernoux *et al.*, 2000b). Rather than localizing to discrete founder cell foci, the *ANT*-expressing cells are distributed in rings encircling *pin1* mutant apices. The expression domain of *CUC2*, which is specifically restricted to organ boundaries in wild-type plants (Aida *et al.*, 1997), is also expanded in *pin1* mutant apices and largely overlaps with that of *ANT* (Vernoux *et al.*, 2000b). Thus, *pin1* mutants are defective both in lateral organ primordia separation and outgrowth, phenotypes that may be caused by insufficient auxin efflux to

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Figure 2.9 Model for the role of *PIN-FORMED1* (*PIN1*) and auxin transport in *Arabidopsis* organ formation. As soon as cells leave the meristem, primordium genes, *AINTEGUMENTA* (*ANT*) and *PIN1*, are upregulated. Directional transport through the *PIN1* transporter causes the accumulation of auxin in the incipient primordium, which inhibits boundary identity via downregulation of *CUP-SHAPED COTYLEDONS2* (*CUC2*) and also stimulates primordium outgrowth together with genes such as *ANT*. (Based on Vernoux *et al.*, 2000. *Development*, **127** 5157-5165, with permission from the Company of Biologists Ltd.)

primordia initiation sites. Consistent with this hypothesis is the observation that inhibition of auxin polar transport blocks leaf formation from the tomato vegetative meristem and causes a phenocopy of the *pin1* mutant phenotype, while exogenous application of the auxin indole-3-acetic acid (IAA) restores leaf formation to tomato pin-like apices and flower formation to *pin1* apices (Reinhardt *et al.*, 2000). Based on these data, a model has been proposed for *PIN1* regulation of organ separation and outgrowth (figure 2.9). According to this model, upregulation of *PIN1* transcription occurs as soon as organ founder cells leave the meristem, leading to auxin accumulation in the initiating organ primordium through the PIN1 transporter. Auxin accumulation establishes a boundary around the primordium by causing *CUC2* downregulation in the organogenic region, and also stimulates outgrowth of the primordium in combination with genes such as *ANT* (for further discussion, refer to chapter 6 of this volume).

2.5 Relation of functional domains to cytologically and anatomically defined domains

2.5.1 Domain-restricted gene expression patterns

Until recently, our understanding of the vegetative shoot apical meristem as a structure that is actively organized into discrete cell layers and zones has been based mainly upon histological and anatomical studies. The interpretation of the histological and cytological data supporting the tunica-corpus and apical zonation models has generated much debate, as has the significance of the observed patterns with respect to the overall functions of the meristem (Steeves and Sussex, 1989). Clonal analysis of chimeric plants has demonstrated that in seed plants, particularly angiosperms, the stratified nature of the shoot apex has developmental relevance in that certain types of tissues are derived largely from a single cell layer (Satina *et al.*, 1940). Further attempts to test the validity of these models have used the tools of molecular biology to identify genes whose function is restricted to a single region of the meristem. Molecular data, accumulated mainly from expression analysis in angiosperms, indicate that the meristem layering and zonation patterns defined by prior anatomical and cytological analysis generally reflect a spatial regulation of gene expression that appears to organize the vegetative shoot apex into discrete functional domains.

A pioneering study by Fleming and co-workers (Fleming *et al.*, 1993) characterized a number of genes that have discrete mRNA expression patterns in the tomato vegetative shoot apical meristem. One gene encoding an arginine decarboxylase and a second encoding a dUTPase were found to be transcribed predominantly in the corpus layers of the meristem. Conversely, two other genes, the first encoding a lipid transfer protein (LTP) and the second encoding a polyphenoloxidase, had elevated expression levels in the L1 cell layer of the

overlying tunica. This study revealed that the tomato meristem is separated into two compartments, the epidermis and the cells underlying it, that can be distinguished based on mutually exclusive patterns of gene expression. Given that the epidermal cell layer is a highly specialized structure that acts both as a barrier to and a site of interaction with the environment, it is not surprising that plants have evolved a genotypically distinct cell population to respond to signals and stresses to which the underlying cells are not exposed.

Several genes of *Arabidopsis* also have exclusive L1-layer-specific gene expression patterns. The *PDF1* gene, which encodes a putative cell wall component, is transcribed initially in the embryonic protoderm, which generates the L1 layer of the meristem, and is later detected in the L1 layer of the vegetative meristem and organ primordia but not in the epidermis of mature organs (Abe *et al.*, 1999). Transcripts from the *FIDDLEHEAD (FDH)* gene are also detected in the vegetative meristem exclusively in the L1 cell layer (Yephremov *et al.*, 1999). Loss-of-function *fdh* mutations cause the postgenital fusion of the epidermis of lateral organs and a reduction in the number of rosette leaf trichomes, which are produced from single L1 cells (Lolle *et al.*, 1992, 1997; Yephremov *et al.*, 1999). *FDH* encodes a protein with similarity to condensing enzymes involved in lipid biosynthesis, suggesting that *FDH* is an L1-specific effector molecule that affects the lipid composition of the epidermis in such a way as to prevent inappropriate cell adherence in response to contact. In fact, all of the above-mentioned genes can be largely considered to be effector genes that carry out the specific enzymatic or structural functions in the cells in which they are expressed.

Putative regulatory genes with region-specific expression patterns in the shoot apical meristem are also being discovered (figure 2.10). Several members of the plant-specific homeobox-leucine zipper (HD-ZipIV) gene family with expression patterns that define subdomains of the vegetative meristem have been isolated in maize and *Arabidopsis*. Three *ZmOCL* genes (*ZmOCL1*, 4 and 5) have L1-layer-specific expression patterns in the vegetative apex, while *ZmOCL3* is confined to the epidermal layer of mature organs (Ingram *et al.*, 2000). Therefore, a subset of homeodomain transcription factors may be responsible for maintaining the identity of cells in specific domains of the vegetative shoot apical meristem. Transcripts from another maize HD-ZipIV family member, *ZmOCL2*, are largely restricted to a cell layer immediately beneath the L1 in vegetative and floral meristems (Ingram *et al.*, 2000). In *Arabidopsis* or other dicotyledonous plants, this region would correspond to the L2 subepidermal cell layer, but previous clonal analysis of the monocotyledonous maize meristems has indicated the existence of only a single tunica layer. This result indicates that three layers of stratification, an epidermal layer, a subepidermal layer and a corpus, are present in maize meristems, at least during later stages of development. Thus, the identification of molecular markers of shoot apical meristem domains has contributed to an important refinement of the layering

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Figure 2.10 Localization of *NTH* mRNAs in the vegetative meristem of tobacco plants. Median longitudinal sections through vegetative shoot apices were probed to detect one of four genes. *NTH15* (a) and *NTH1* (b) are mainly expressed in the corpus, while *NTH9* (d) is expressed in the rib zone (rm) and *NTH20* (f) is expressed in the peripheral zone (pz) but not in the central zone (cz). The arrowhead indicates the leaf primordium (lp) initiation site (internal scale bars = 50 μ m). (Reprinted with permission from Nishimura *et al.*, 1999. *Plant Journal*, **18** 337-347.)

model with respect to one plant species that was not revealed by prior histological analyses.

A homologous HD-ZipIV gene in *Arabidopsis*, *ATML1*, is transcribed in the protoderm from the onset of its formation in the embryo, and during the vegetative phase, *ATML1* mRNA is detected exclusively in the L1 of the meristem and initiating lateral organs (Lu *et al.*, 1996). Thus, *ATML1* may play a role in the specification as well as the maintenance of the L1 cell layer. In other organisms, such as *Drosophila*, multiple homeobox genes are expressed in overlapping patterns in the developing embryo (Ingham, 1988; McGinnis and Krumlauf, 1992; Gehring *et al.*, 1994). The combinatorial activities of these homeodomain proteins act to pattern the epidermis during embryonic development. By analogy to these other organisms, it has been proposed that the activity of members of plant homeodomain families in specific layers or domains establishes the morphogenetic boundaries of positional information required to control cell identity and pattern formation in the vegetative shoot apex (Nishimura *et al.*, 1999). *ATML1* expression precedes and is spatially coincident with *PDF1* expression in the protoderm and meristem L1 cell layer, making *PDF1* a candidate target of region-specific homeodomain protein regulation. *ATML1* and *PDF1* may therefore illustrate an evolutionarily conserved phenomenon in which the spatially-restricted expression of transcription factors, such as those encoded by homeobox genes, is utilized to control the localized

activation of effector molecules and thereby specify discrete functional domains within the vegetative shoot apex.

Analysis of gene expression patterns in angiosperm meristems has also provided molecular evidence supporting the zonation model (figure 2.10). *CLV3* gene transcripts are localized to the central region of the shoot meristem of *Arabidopsis* (Fletcher *et al.*, 1999), where *CLV3* signals from the stem cells to restrict meristem activity mediated by the underlying *WUS*-expressing cells. The tobacco class I *knox* homeobox gene, *NTH20*, is expressed in the peripheral region of the vegetative shoot apex, as is the gene encoding the tomato ribosomal protein, *L38* (Fleming *et al.*, 1993). Messenger RNA from the tobacco *NTH9* gene, which is related to *NTH20*, is localized preferentially to cells corresponding to the rib zone (Nishimura *et al.*, 1999). This differential gene expression reveals the presence of three genetically distinct populations of cells in the shoot apical meristem, whose domains correlate with those defined by cytological and anatomical analysis. Furthermore, the expression of distinct class I *knox* genes may specify the identity of the peripheral and rib zones, providing positional information to cells as they pass from the central zone, marked by *CLV3* expression, into regions where the differentiation process begins.

Several genes associated with cell division also display region-specific localization patterns consistent with the notion that the meristem zones correspond to different functional domains. The *cyclin D3a* gene of *Antirrhinum* is expressed in the peripheral region but not in the central region of the vegetative apex, suggesting that differences in the rate of cell proliferation previously observed between these areas may involve the differential expression of *D-cyclin* genes (Gaudin *et al.*, 2000). H3 and H4 histone genes from *Arabidopsis* and tomato, respectively, are also preferentially localized to the peripheral zone of the mature vegetative apex (Medford *et al.*, 1991; Brandstadter *et al.*, 1994). Histone gene expression is an indicator of DNA replication, so these data are also consistent with prior histological observations that the cells on the periphery of the meristem divide more rapidly than those in the center. These data also suggest that the molecular basis for localized differences in cell division rates within the vegetative meristem may involve differences in the rate of entry into, or progression through, the cell cycle (for further discussion, refer to chapter 8 of this volume).

There are several important factors to be considered when making direct comparisons between regional gene expression patterns that subdivide the meristem, and the cytologically and anatomically defined meristem domains. The first is that, with a few exceptions, such as *CLV3*, most of the genes that are expressed in domain-specific expression patterns in the vegetative shoot apical meristem are also transcribed in differentiated tissues, including roots (Fleming *et al.*, 1993; Nishimura *et al.*, 1999; Ingram *et al.*, 2000). Thus, genes that are transcribed in restricted domains of the meristem are not necessarily meristem-specific

genes, and do not necessarily encode products that perform functions that are exclusive to the meristem. The second consideration is that the mRNA expression pattern of a gene does not necessarily delimit the entire functional domain of the encoded product. For example, *kn1* mRNA is localized to the corpus of the maize vegetative meristem and excluded from the L1 epidermal layer, but KN1 protein is detected throughout the entire meristem, including the L1 (Jackson *et al.*, 1994). One must therefore be cautious when extrapolating the presence of functional domains in the vegetative meristem based solely on observations of region-specific gene transcription profiles. While it is clear from the molecular evidence that genetically distinct cell layers and zones are present in the shoot apical meristem, concrete evidence that the histologically defined domains reflect discrete, developmentally relevant functional programs of gene activity awaits the localization of the proteins in question.

2.5.2 *Coordination between meristem domains*

The fact that genetically separable cell layers and domains exist in the vegetative meristem indicates that the various activities of the meristem must be coordinated both within and between them. One way in which coordination between different meristem regions may be achieved is through symplastic trafficking. Experiments using fluorescent tracer labeling of shoot apices have revealed that the tunica layers of both *Arabidopsis* and birch exhibit symplastic connectivity. This symplastic connectivity occurs via primary and secondary plasmodesmata that link adjoining cells, providing intercellular connections that may enable regulated movement of signaling molecules via the cytoplasm (Rinne and van der Schoot, 1998; Gisel *et al.*, 1999). The distribution and the size exclusion limit of plasmodesmata have been observed to vary between different developmental stages (Lucas *et al.*, 1993), suggesting that intercellular trafficking is regulated and plays an important role in the coordination of meristem domains during plant development.

The corpus region of the meristem appears to form a separate symplastic domain from the tunica, but it has been proposed that secondary plasmodesmata maintain a connection between the corpus and the tunica in order to integrate the three clonal cell groups (van der Schoot and Rinne, 1999). Small protein and peptide signals, such as CLV3 (Fletcher *et al.*, 1999) and systemin (Narvaez-Vasquez *et al.*, 1995), are also predicted to flow between the symplastically isolated domains, unrestricted by plasmodesmatal barriers due to their movement through the apoplastic space between the cell walls themselves, in order to facilitate communication between the various regions of the meristem. Dye tracer injection experiments using apices of birch indicate that the central and peripheral regions of the meristem are also symplastically isolated from one another (Rinne and van der Schoot, 1998). Intercellular coupling between the

two regions was not detected in these experiments except for a transient period, possibly during the initiation of each leaf primordium. Although it is not clear whether these two compartments coincide with the classically defined central zone and peripheral zone, they do reveal a potential mechanism through which the behavior of cells within a meristem domain can be organized via the localized symplastic trafficking of signaling molecules.

2.6 Summary

The vegetative meristem is a highly dynamic structure that undergoes continuous organogenesis, while simultaneously maintaining a reservoir of undifferentiated stem cells that constantly replenish those cells lost to organ formation. The vegetative meristem is organized into cytohistologically separable domains and clonally distinct cell lineages. However, the cells derived from these lineages do not have fixed developmental fates and instead rely on positional information to cue their patterns of proliferation and differentiation. Homeodomain transcription factors are critical for promoting and maintaining meristem cell activity, but are counterbalanced by stem cell restricting functions that limit meristem cell accumulation. Downregulation of meristem promoting factors on the meristem periphery appears to be essential for organogenesis, and requires another class of transcription factors that are specifically expressed in organ founder cells. These genes, in turn, may directly or indirectly activate downstream effector molecules, triggering the transition of these cells into specific differentiation pathways that culminate in leaf formation. The hormone, cytokinin, also appears to promote meristem cell function, while localized auxin accumulation on the meristem periphery is required for morphogenesis and the establishment of boundaries between the primordium and the vegetative shoot apex. Thus, a major theme of vegetative meristem function is the subdivision of the apex into genetically and functionally distinct domains, each expressing a unique combination of regulatory and effector genes. Maintenance of these functional regions appears to depend, to a large extent, on negative gene interactions, which act to stabilize the distinct identities of neighboring domains. However, communication between the different cell layers and domains is essential to coordinate proper meristem growth and function, and is likely to involve both cytoplasmic and apoplastic signaling factors.

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3 Shoot apical meristem formation in higher plant embryogenesis

Mitsuhiro Aida and Masao Tasaka

3.1 Introduction

Plants continue to produce organs and tissues throughout their lifetime. This is in contrast to animals, in which most organogenic events are confined to embryogenesis. The shoot apical meristem (SAM), located at the apical end of the axis, plays a central role in shoot organ production. Cells in the SAM have characteristics of stem cells, including being undifferentiated and having the potential to divide for self-renewal as well as producing cells that are incorporated into differentiating organ primordia. Most above-ground organs are ultimately derived from the SAM. Thus, to understand how the SAM is formed and how it functions has been a central theme in studies of plant development.

In angiosperms, the SAM has a layered structure that reflects patterns of cell division within it. The outer layer, the tunica, consists of one or more layers of cells that divide only anticlinally (i.e. new cell walls form perpendicular to the surface). Enclosed by the tunica is the corpus, in which cell division occurs in various directions. Besides its tunica-corpus structure, the SAM can be partitioned into three functionally distinct zones: the central zone (CZ), the peripheral zone (PZ) and the rib meristem (RM). The CZ is located at the summit and provides undifferentiated cells to the surrounding PZ and RM. The PZ and RM, in which cells divide more rapidly, generate cells used in lateral organs and inner tissues of stems, respectively.

The SAM is initially formed during embryogenesis. Starting from a single zygote, plant embryos develop a simple structure with a distinct pattern consisting of the SAM, cotyledon, hypocotyl, root and the root apical meristem (RAM) along the apical-basal axis. During this process, the embryo initially establishes the apical-basal axis with distinct polarity. Then, each of the embryonic organs and meristems differentiates at appropriate positions along the axis. Plant embryogenesis can be viewed, essentially, as the initial differentiation of the shoot and root fates. This differentiation is totally dependent on the apical-basal polarity of the embryo. Once each of the fates is established, it is maintained throughout the life of the plant. In general, shoots do not produce roots and roots do not produce shoots.

This chapter focuses on SAM formation during embryogenesis of higher plants. Following a brief summary of the morphological events related to SAM formation in embryogenesis, several early events are discussed, such as establishment of the apical-basal polarity and formation of several embryonic organs along the axis. A discussion of how the apical region of the embryo is subdivided into more highly organized regions including the SAM, follows. Finally, the chapter focuses upon several important regulators of SAM development and there is a discussion of how these genes are activated during embryogenesis. Previous reviews have covered several aspects of embryogenesis, including pattern formation (Laux and Jürgens, 1997), RAM formation (Scheres and Heidstra, 1999), induction of embryo development (Harada, 1999) and endosperm development period (Grossniklaus *et al.*, 2001).

3.2 Morphological events during embryogenesis and SAM formation

Classical descriptions of morphogenic events associated with angiosperm embryogenesis have been described previously in several reviews (Steeves and Sussex, 1989; West and Harada, 1993). The embryogenesis of *Arabidopsis*, from which a large part of the molecular genetic data described in this chapter has been obtained, has also been described previously (Mansfield and Briarty, 1991a, 1991b; Jürgens and Mayer, 1994; Torres-Ruiz, 1998). Here, several morphological features that are involved in polarity, patterns of cell division and development of SAM organization are summarized.

The embryo of higher plants develops in the ovule (figure 3.1A). Establishment of polarity is a crucial early event in embryogenesis. In most plant species, an apical-basal polarity inherited by the zygote is already apparent in the egg (Mansfield and Briarty, 1991a). In *Arabidopsis* and other crucifers, for

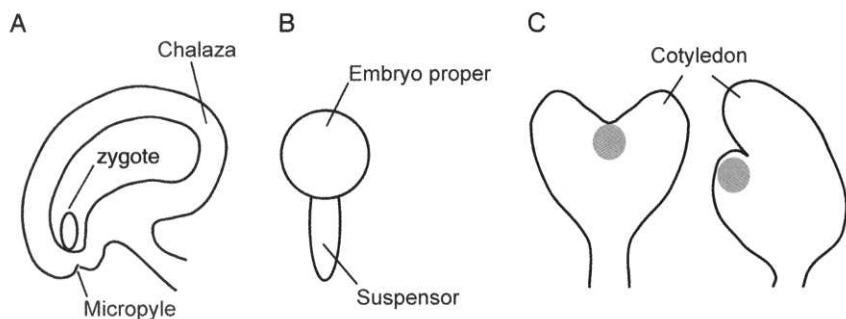


Figure 3.1 Embryogenesis and shoot apical meristem (SAM) formation in angiosperms. A: Position of the zygote in the ovule. B: The embryo proper and the suspensor. C: Positions at which the SAM is formed in dicotyledons (left) and monocotyledons (right).

example, the nucleus is located towards the apical end of the egg and zygote, while a large vacuole is observed at the basal end (Schulz and Jensen, 1968; Sumner and Caesele, 1989; Mansfield and Briarty, 1991a). In contrast, the nucleus of the unfertilized eggs in *Papaver* and maize is located towards the basal side, but then shifts to the apical end at fertilization (Olson and Cass, 1981; van Lammeren, 1981).

In many angiosperms, the zygote divides transversely to produce a small apical cell and a large basal cell. After several rounds of cell division, the apical cell mainly gives rise to the embryo proper, while the basal cell gives rise to the suspensor (figure 3.1B). The embryo proper will develop into the plant body. The suspensor is an appendage that is proposed to provide nutrients and growth regulators to the developing embryo proper, which eventually degenerates during embryogenesis. Initial division patterns of the zygote and its descendents may be regular or irregular, depending on the species. Crucifers, including *Arabidopsis*, display a highly stereotyped pattern of cell division (West and Harada, 1993), while other species, such as maize or cotton, display no obvious regularity (Pollock and Jensen, 1964; Poethig *et al.*, 1986).

In dicotyledons, the SAM develops between two cotyledon primordia in the central and apical region of the embryo (figure 3.1C, left), while in monocotyledons, the SAM develops laterally at the base of a single cotyledon (figure 3.1C, right). SAM development during embryogenesis has been studied in various angiosperms using morphological criteria, such as tunica-corpora organization or histological zonation (Steeves and Sussex, 1989; Lyndon, 1998). These studies have revealed that the times at which these features are established vary among species (Lyndon, 1998). For example, histological zonation is already apparent before the initiation of cotyledons in species such as *Downingia* or *Stellaria* (Pritchard, 1964; Kaplan and Cooke, 1997), while no such staining pattern can be observed at the heart stage embryo of *Capsella* although cotyledon primordia are already visible (Kaplan and Cooke, 1997). In *Arabidopsis*, the tunica-corpora organization becomes complete before the emergence of the first foliage leaf primordia (Barton and Poethig, 1993), while in pea, the division pattern in the tunica layer is not fully established until several leaf primordia have been formed (Reeve, 1948).

3.3 Formation and maintenance of the polarity along the apical-basal axis

Differences between the two major organ systems, the shoot and root, ultimately originate from the polarity along the apical-basal axis in the embryo. Although the polarity is already apparent in the zygote or even in the egg before fertilization, it can be disturbed by zygotic mutations or physiological perturbations. Therefore, the initial apical-basal polarity needs to be fixed after fertilization.

Although little is known about how the initial polarity is generated, recent genetic and physiological analyses suggest that the fixation of the pre-existing axis involves directional vesicle transport and polar auxin transport.

3.3.1 *Origin of the apical-basal polarity*

The direction of the apical-basal axis of the egg and zygote is invariably along the chalaza-micropyle axis of the ovule (figure 3.1A). This regularity arises largely from a patterning process during female gametogenesis, in which regular sets of formative divisions of haploid cells result in the formation of the embryo sac with stereotyped arrangements of cells (Drews *et al.*, 1998). The strict correlation between the direction of the egg cell and that of the ovule raises the possibility that maternal information plays an important role in establishment of the apical-basal polarity of the embryo (Laux *et al.*, 1996). Such information, however, may not be strictly required, because plant cells can establish the apical-basal polarity without maternal tissues, as is demonstrated by somatic embryogenesis. Nevertheless, it is still reasonable to postulate that maternal information does provide a positional cue that promotes a preferential polarity of the egg in seed plants.

In *Drosophila*, the isolation of many maternal effect mutants has elucidated the important role of maternal tissues for early embryo patterning, including polarity determination (Johnston and Nüsslein-Volhard, 1992). In contrast, there are only a few examples of maternal effect mutants in plants. One of these, the *short integument1* (*sin1*) mutant of *Arabidopsis* (Ray *et al.*, 1996), originally reported to affect ovule formation and flowering time, also affects embryo development in mutant ovules. Defects are variable, ranging from embryos with reduced SAM to chaotic masses of unorganized tissues. The latter phenotype may reflect abnormalities in very early stages of embryogenesis, although detailed examination of embryogenesis in *sin1* has not yet been reported. The phenotype of *sin1* embryos is dependent on the genotype of the maternal tissue, but not on the zygotic tissue, showing a maternal effect of the *sin1* mutations. It is still not clear, however, whether the *SIN1* gene is truly required for normal development of the zygotic embryo, or whether the abnormalities of embryos are merely caused by a secondary effect of abnormal development of the mutant ovule.

3.3.2 *Establishment of the apical-basal polarity*

The *GNOM/EMBRYO DEFECTIVE30* (*GN/EMB30*) gene of *Arabidopsis* is required for the fixation of the apical-basal polarity, with the earliest phenotype of the *gn/emb30* mutant detected at the first division of the zygote (Mayer *et al.*, 1993). The division plane of *gn/emb30* is displaced to a position that separates the zygote into cells of the same size, whereas the wild-type zygote divides into cells of two different sizes. Later in embryogenesis, the *gn/emb30* mutation

displays an altered apical-basal polarity, as is revealed by variable expression of an apical-specific marker gene, *Arabidopsis thaliana* *Lipid Transfer Protein1* (*AtLTP1*) (Vroemen *et al.*, 1996). In severe cases, *gn/emb30* develops a ball-shaped embryo in which *AtLTP1* is expressed ubiquitously. These phenotypes suggest that *GN/EMB30* is required to stabilize the apical-basal polarity of the embryo as early as the first division of the zygote.

Cellular organization in *gn/emb30* embryos has been examined using a protein marker for cell polarity (Steinmann *et al.*, 1999). PIN-FORMED1 (PIN1), a member of the putative auxin efflux carrier (Gälweiler *et al.*, 1998). In the wild-type, PIN1 is localized in a polar fashion from the mid-globular stage, accumulating at the basal boundary of four elongated inner cells. At the heart stage, PIN1 localizes to the basal boundary in cells of the provascular tissue, while the opposite polarity is seen in the epidermis. Individual cells that show polar localization of PIN1 are coordinately aligned depending on their relative positions. By contrast, although polar localization of PIN1 can be observed in cells of *gn/emb30* embryos, orientations of polarity of individual cells are not correctly aligned with one another, being uncoupled from their positions (Steinmann *et al.*, 1999). These observations indicate that *GN/EMB30* is required for coordinated cell polarity at the tissue level.

What is the relationship between the altered pattern of auxin efflux carrier PIN1 and the axis defect in *gn/emb30*? The pattern of PIN1 distribution in the wild-type globular embryo indicates that the direction of auxin transport is along the apical-basal axis (Steinmann *et al.*, 1999). Furthermore, inhibition of auxin transport in *Brassica juncea* embryos cultured *in vitro* at the globular stage causes complete loss of the apical-basal polarity, resulting in a phenocopy of *gn/emb30* embryos (Hadfi *et al.*, 1998). These results suggest that polar auxin transport along the apical-basal axis promotes the establishment of apical-basal polarity. Therefore, it is possible that the altered pattern of PIN1 in *gn/emb30* perturbs auxin transport and thereby causes the axis defect.

The relationship between *GN/EMB30* and PIN1 can generate a positive feedback loop, in which *GN/EMB30* promotes coordinated polar localization of PIN1 according to a pre-existing apical-basal polarity. In turn, coordinated PIN1 localization strengthens the polarity by promoting polar auxin transport along the apical-basal axis. The observed gradual establishment of coordinated PIN1 localization, as well as the stochastic axis defects in *gn/emb30* embryo, supports this model.

The *GN/EMB30* gene encodes a protein homologous to yeast *Gea1p* and *Gea2p*, which are guanine nucleotide exchange factors (GEFs) for G proteins of the ADP-ribosylation factor (ARF) family required for vesicle transport (Shevell *et al.*, 1994; Busch *et al.*, 1996; Peyroche *et al.*, 1996). GEFs that act on ARFs (ARF GEFs) are subdivided into small and large families. Large ARF GEFs, including *Gea1p* and *Gea2p*, can be specifically inhibited by the fungal metabolite, brefeldin A (BFA) (Chardin *et al.*, 1996; Peyroche *et al.*,

1996; Sata *et al.*, 1998). The *GN/EMB30* gene can complement the yeast *gea1* and *gea2* mutants, and recombinant *GN/EMB30* protein shows an ARF GEF activity that can be blocked by BFA treatment (Steinmann *et al.*, 1999). These findings indicate that *GN/EMB30* is a BFA-sensitive ARF GEF that is involved in vesicle transport, and so may promote polar localization of specific proteins through directional vesicle transport within individual cells.

Is PIN1 cellular localization directly regulated by GN/EMB30-dependent vesicle transport? BFA treatment can completely block polar localization of PIN1 and results in its even distribution along the entire cell surface, suggesting that BFA-sensitive ARF GEF is indeed involved in polar localization of PIN1 (Steinmann *et al.*, 1999). However, cells of *gn/emb30* still show PIN1 polar localization, although they are not coordinated at the tissue level. This observation suggests either that GN/EMB30 may not be involved in this process, or that the polar localization of PIN1 in *gn/emb30* reflects activities of other BFA-sensitive ARF GEFs that act redundantly with GN/EMB30.

Directional vesicle transport also appears to play an important role in establishment of the apical-basal polarity of the brown algae, *Fucus* (Shaw and Quatrano, 1996; Quatrano and Shaw, 1997; Belanger and Quatrano, 2000). The *Fucus* zygote asymmetrically divides to produce two cells with different fates, thallus and rhizoid. The axis of the polarity is first oriented by external cues, such as sperm entry, light or gravity. The fixation of this axis occurs shortly before the first division of the zygote and is thought to require actin filaments and materials associated with the cell wall. Prior to the first division, the basal end of the zygote accumulates Golgi-derived secretory vesicles that are thought to deposit cell wall materials. BFA treatment of the *Fucus* zygote prevents accumulation of the secretory vesicles and axis fixation. These findings suggest that BFA-sensitive vesicle transport, which is probably mediated by actin filaments, is required to fix polarity through localized deposition of cell wall materials.

3.4 Fate specification along the apical-basal axis

As embryogenesis proceeds, specification of embryonic organs along the apical-basal axis occurs sequentially. First, the early proembryo is separated into two different fates, the embryo proper and the suspensor. The embryo proper is then further subdivided into the SAM, cotyledons, hypocotyl, root and the RAM. In each case, cellular interactions, rather than cell lineage-based mechanisms, appear to play important roles in fate decisions.

3.4.1 Fate specification of the embryo proper and the suspensor

The first division of the *Arabidopsis* zygote is asymmetric, since both morphology and fates of the two daughter cells are different (figure 3.2). The apical cell contributes predominantly the embryo proper, while the basal cell mainly

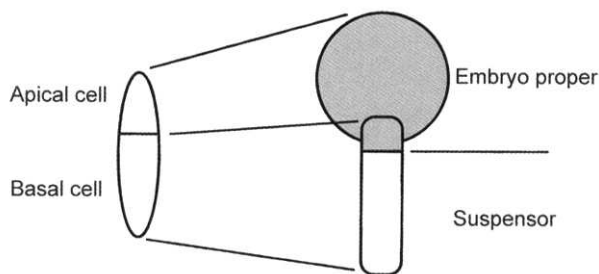


Figure 3.2 Origin of the embryo proper and the suspensor in *Arabidopsis*.

develops into the suspensor. However, the fate of the basal cells is not fixed, at least at the first division, because one of its derivatives, the hypophysis, will eventually give rise to the root cap of the embryo proper.

The *Arabidopsis twin* mutants (*twn1* and *twn2*), frequently develop secondary embryos from the basal cell derivatives, suggesting that the basal cell retains the capacity to change its fate to the embryo proper (Vernon and Meinke, 1994; Zhang and Somerville, 1997). In *twn2*, the primary defect occurs in the apical cell, which stops development immediately after the first division of the zygote. This developmental arrest appears to be caused by altered expression of a housekeeping gene, *valyl-tRNA synthetase*, as a result of a T-DNA insertion into the 5' untranslated region of the gene. An important feature of this mutant is that the formation of secondary embryos from the basal cell lineage always starts after the arrest of the apical cell. A similar situation is observed in the *suspensor* (*sus*) and *raspberry* mutants, in which the embryo proper arrests at certain stages of development and embryo-like structures develop from the basal lineage after the arrest of the embryo proper (Schwartz *et al.*, 1994; Yadegari *et al.*, 1994). Based on these findings, it is proposed that the alternative fate of the basal cell and its descendants is suppressed by the apical cell and embryo proper in normal development, and defects in the apical cell lineage cause failure of this suppression.

In the *twn1* mutant, twinning can occur without developmental arrest of the embryo proper (Vernon and Meinke, 1994). One possible explanation is that the *TWN1* gene is involved in a signalling pathway that restricts the basal descendants to a suspensor cell fate. Molecular cloning of *TWN1* will permit tests of this hypothesis.

Arabidopsis LEAFY COTYLEDON1 (LEC1) and *FUSCA3 (FUS3)*, which regulate many aspects of embryogenesis, such as cotyledon identity, desiccation tolerance and repression of precocious germination, are also involved in preventing suspensor cells from adopting an embryo fate (Parcy *et al.*, 1997; Lotan *et al.*, 1998). Suspensor cells of the *lec1* or *fus3* single mutants display irregular patterns of cell division. This phenotype is significantly enhanced

in the double mutant, leading to the formation of secondary embryos from suspensor cells. These results suggest that *LEC1* and *FUS3* have overlapping functions that limit the basal cell fate. *LEC1* encodes a protein homologous to the CCAAT box binding transcription factor, HAP3. Overexpression of *LEC1* causes ectopic formation of embryos from vegetative cells, suggesting that *LEC1* promotes the embryo fate. The *LEC1* transcript is normally accumulated both in the embryo proper and the suspensor, and thus the simple expression of *LEC1* in the suspensor does not by itself distinguish the suspensor and embryo fates. It is possible that a *LEC1*-dependent inhibitory signal from the embryo proper may block embryo inducing activity of *LEC1* in suspensor cells.

The phenotype of *tnl1* and *lec1 fus3* embryos provides an interesting insight into polarity determination. The apical-basal polarity of the secondary embryo does not always coincide with that of the original embryo (Vernon and Meinke, 1994; Lotan *et al.*, 1998). A secondary embryo of *tnl1* and *lec1 fus3* is always flanked by two suspensors at opposite sides, while an original embryo is flanked by a single suspensor at its basal side. These observations raise the possibility that the relative position of the embryo proper and the suspensor helps to establish the apical-basal polarity. The abnormal position of secondary embryos of *tnl1* and *lec1 fus3* relative to the suspensor suggests that these genes may be required to interpret a positional cue.

3.4.2 Fate specification within the embryo proper

The early embryo of *Arabidopsis* can be divided into three regions, apical, central and basal, which are derived from the upper tier, lower tier and hypophysis of the octant stage embryo respectively (figure 3.3) (Jürgens, 1995). The

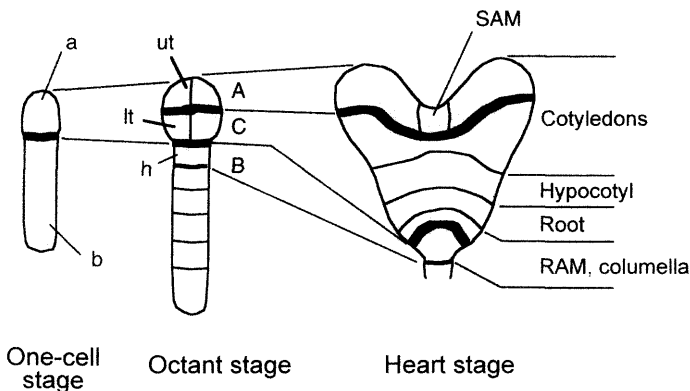


Figure 3.3 Fate map of the *Arabidopsis* embryo. Abbreviations: A: apical region. B: basal region. C: central region. a, apical cell; b, basal cell; h, hypophysis; lt, lower tier; ut, upper tier; SAM, shoot apical meristem; RAM, root apical meristem. (Taken from Willemsen *et al.*, 1998.)

embryo will ultimately subdivide into the SAM, cotyledons, hypocotyl, root and the RAM. To gain insight into the interactions between the three early regions and their final fates, a fate map of the heart stage embryo was constructed based on detailed histological and clonal analyses (Scheres *et al.*, 1994). This analysis suggests that the three regions that develop early in development do not have a simple correspondence to the prospective organs or meristems. For example, cotyledons originate not only from cells of the apical region but also from those of the central region. Similarly, the RAM is derived from cells of both the central and basal regions. These findings suggest that cell fates in the three regions are not separated at the octant stage and that determination of organ fates as well as positioning of organ boundaries occurs later in embryogenesis.

Consistent with this view, clonal analysis indicates that clones that are presumed to arise from cells at the same position in early embryos show variable boundaries in later stages of development (Scheres *et al.*, 1994). Therefore, although the pattern of cell division is very regular in the *Arabidopsis* embryo, final cell fates are not strictly determined by a cell-lineage-dependent mechanism, but are strongly influenced by cellular interactions that play an important role in fate decisions. The *fass* (*fs*) mutant provides additional support for this view (Torres-Ruiz and Jürgens, 1994; Traas *et al.*, 1995). The regularity of cell division patterns is completely disrupted in *fs*, leading to the formation of extremely compressed embryos. However, despite these changes in cell division patterns, *fs* can differentiate all of the embryonic organs and meristems at appropriate positions. In view of these findings, the importance of the three cellularly defined regions of the early *Arabidopsis* embryo is ambiguous. Nevertheless, they are still useful because each of these regions is related to the final subdivisions of the embryo to some extent, thus facilitating the description of mutant phenotypes and gene expression patterns.

Several mutants that affect formation of specific embryonic organs and meristems have been isolated. The *topless* mutant shows defects in formation of the SAM and cotyledons, which are mainly derived from the apical region (Evans and Barton, 1997; Long *et al.*, 1999). The phenotype of *tpl* is variable, and embryos with the most severely affected mutants develop a root instead of cotyledons and the SAM. Embryos of *tpl* fail to express the SAM-specific genes, *SHOOT MERISTEMLESS* (*STM*) and *UNUSUAL FLORAL ORGANS* (*UFO*) (see section 3.6), but express a root specific marker in the apical region. These results suggest that the *TPL* gene is required both to specify the fate of the apical region of the embryo and to suppress the root fate.

Mutations in the *MONOPTEROS* (*mp*) gene cause deletion of the hypocotyl and root (collectively referred to as the embryo axis), which are derived from the central and basal regions (Berleth and Jürgens, 1993). Occasionally, *mp* mutants produce adventitious roots from seedlings or tissue cultures, suggesting that the *MP* gene is not required for the formation of the root per se. During embryogenesis, *mp* mutants fail to form elongated provascular cells

in the central region of the embryo. In normal development, this region mainly gives rise to the embryo axis. Defects in vascular formation are not limited to embryogenesis: vascular tissues are significantly reduced and are incompletely connected in all organs examined, including cotyledons, leaves and stems (Przemeck *et al.*, 1996). These observations suggest that the *MP* gene is required to promote vascular strand continuity both in embryonic and postembryonic development.

Physiological studies have suggested that the coordinated elongation of vascular cells along a specific direction (referred to as cell axialization) requires a flow of auxin along the direction of elongation (Sachs, 1991; Scheres and Berleth, 1998). In *mp* mutants, polar auxin transport is reduced and the inflorescence stems show morphological defects reminiscent of plants treated with inhibitors of auxin transport (Przemeck *et al.*, 1996), raising the possibility that *MP* is involved in an auxin-mediated cell axialization process. The *MP* gene encodes AUXIN RESPONSE FACTOR5 (ARF5), a possible transcription factor that can bind to short-conserved DNA sequences called auxin response elements (AuxREs), which are found in a certain class of auxin inducible genes (Hardtke and Berleth, 1998; Ulmasov *et al.*, 1999b). In addition to DNA binding, the MP protein can activate transcription in carrot cells (Ulmasov *et al.*, 1999a). These findings suggest that MP is a transcription factor that mediates the auxin signalling required for cell axialization.

Two other auxin responsive mutants, *bodenlos* (*bdl*) and *auxin resistant6* (*axr6*), also show defects both in the cell axialization process and formation of the embryo axis (Hamann *et al.*, 1999; Berleth *et al.*, 2000; Hobbie *et al.*, 2000). The strict correlation between these two processes may suggest that cell axialization in the central region of the early embryo is a prerequisite for formation of the embryo axis. Alternatively, auxin signalling pathway(s) that involve(s) the *MP*, *BDL* and *AXR6* genes has a specific role in embryonic axis formation in addition to cell axialization.

Finally, the *hobbit* (*hbt*) mutant fails to develop a functional RAM (Willemsen *et al.*, 1998). In normal development, the hypophysis undergoes stereotyped cell division to form the basal region, and eventually gives rise to the quiescent centre (QC) and columella root cap, while adjacent cells become the initial cells of root tissues in the RAM. By contrast, the basal region of *hbt* mutants shows irregular cell division patterns from the quadrant stage onward. As a result, *hbt* gives rise to seedlings that lack a QC and columella root cap. In addition, *hbt* can neither establish an active RAM nor differentiate lateral root cap cells derived from the central region. Phenotypes associated with the defects in central-derived cells can be detected at significantly later stages than the defects in the basal region. Because defects in *hbt* appear to be limited to the basal region, at least in the early stages of embryogenesis, it is proposed that this region represents a specific developmental compartment in the embryo and that its specification requires *HBT* activity (Willemsen *et al.*, 1998). If so, defects in the RAM and

lateral root cap of *hbt* seedlings may reflect a non-cell-autonomous function of *HBT*. In the active RAM, short-range signals emanating from the QC are thought to be important for the surrounding cells to maintain their stem cell activity (van den Berg *et al.*, 1997). Therefore, it is possible that RAM establishment during embryogenesis may require signals from the basal-derived QC cells, and that *HBT* can affect RAM and lateral root cap formation by promoting QC formation. The cloning and expression analysis of *HBT* should either confirm or refute this hypothesis.

3.5 Elaboration of the apical region of the embryo

The apical region of the embryo is elaborated further, leading to the formation of the SAM and two cotyledons. Recent studies on *Arabidopsis* mutants and gene expression related to this process have suggested that the apical region of the globular stage embryo is subdivided into three zones with different fates (figure 3.4A), including: a region at the centre that gives rise to the SAM, regions at the peripheries that form cotyledons, and regions at the peripheries in which growth is suppressed to form the boundaries between the cotyledons. In this section, the function and expression of several key *Arabidopsis* genes that are involved in formation of these subregions are described and possible positional information that defines the expression pattern of these genes is discussed.

3.5.1 Formation of the SAM and boundaries between cotyledons

The first morphological indication of subdivision in the apical region is the initiation of cotyledon primordia, which occurs at the late globular stage. At

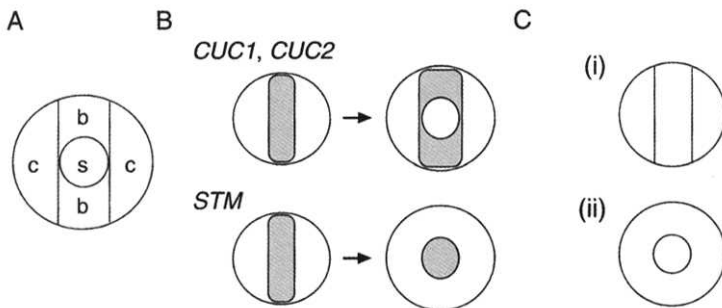


Figure 3.4 Elaboration of the apical region in the *Arabidopsis* embryo. A: Three subregions that develop from the apical region viewed from above. B: Expression patterns of *CUC1*, *CUC2* and *STM*. C: Two patterns in the apical region: (i) bilateral pattern; (ii) radial pattern. Abbreviations: b, boundary between cotyledons; c, cotyledon; s, shoot apical meristem; *CUC1* and 2, *CUP-SHAPED COTYLEDONS 1* and 2; *STM*, *SHOOT MERISTEMLESS*.

this stage, two regions of bulging cotyledon primordia and a depressed region between them can be distinguished. The latter region will be further subdivided into the SAM at the centre and boundaries between cotyledons at the periphery, although they are morphologically indistinguishable at the initiation of cotyledon primordia. This process involves interaction among three genes, *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2* and *STM* (Aida *et al.*, 1999).

CUC1 and *CUC2* are functionally redundant genes and cause a distinct phenotype only when both of the genes are disrupted (Aida *et al.*, 1997). In agreement with this proposed redundancy, the two genes encode highly similar proteins with identity to putative transcription factors (Aida *et al.*, 1997; Takada *et al.*, 2001). *cuc1 cuc2* double mutants fail to develop the SAM. In addition, boundaries between cotyledons, where growth is normally suppressed, grow ectopically and lead to the formation of a fused cup-shaped cotyledon. These results suggest that *CUC1* and *CUC2* are required for SAM formation at the centre and for the suppression of growth at cotyledon boundaries to separate cotyledons (Aida *et al.*, 1997, 1999). Similarly, mutations in the *STM* gene cause loss of a SAM and slight fusion of cotyledons at the base. However, the effect of even strong *stm* mutations on cotyledon fusion is much weaker than *cuc1 cuc2* (Clark *et al.*, 1996; Endrizzi *et al.*, 1996; Long and Barton, 1998; Aida *et al.*, 1999). This observation suggests that *STM* is absolutely required for SAM formation but only partially required for cotyledon separation.

Expression of all three genes begins at the globular stage. Although initial expression patterns of each gene differ slightly when compared with one another, they soon overlap in the stripe region between cotyledon primordia by the early heart stage (figure 3.4B, top) (Long and Barton, 1998; Aida *et al.*, 1999; Takada *et al.*, 2001). This stripe largely corresponds to the presumptive SAM and the boundaries between cotyledons, where the genes are thought to function. By the bent cotyledon stage, when the SAM becomes evident as a bump at the centre, expression of the three genes resolves into two complementary patterns (figure 3.4B, bottom). *STM* is downregulated at the peripheries and becomes restricted to the central SAM, whereas *CUC1* and *CUC2* are downregulated at the centre and become restricted to the region surrounding the centre. These results suggest that subdivision of the stripe region into the SAM and cotyledon boundaries is established by the bent cotyledon stage.

The shift in expression patterns of *CUC1*, *CUC2* and *STM* could be an important event in dividing the stripe region into two regions of different fates, the SAM and cotyledon boundaries. However, there is only limited information about the mechanisms that regulate the expression patterns of these genes. In *stm* embryos, expression patterns of *CUC1* and *CUC2* are altered at the bent cotyledon stage, suggesting that *STM* is required for restriction of *CUC1* and *CUC2* expression (Aida *et al.*, 1999; Takada *et al.*, 2001). This result,

together with the complementary patterns of the *CUC* and *STM* genes at this developmental stage, suggests that *STM* represses *CUC1* and *CUC2* at the centre. These three genes, however, are expressed in overlapping regions at earlier stages, implying that other factors are involved in repression of the *CUC* genes by *STM*.

When *STM* expression has become confined to the centre, it is continuously required for SAM maintenance throughout postembryonic development. Therefore, activation of *STM* expression is a critical step in SAM formation during embryogenesis. As discussed in section 3.6.2, *CUC1* and *CUC2* play an important role in *STM* activation (Aida *et al.*, 1999; Takada *et al.*, 2001).

3.5.2 Cotyledon formation

Several genes are expressed in cotyledon primordia. Some of these are expressed in whole cotyledon primordia (Elliott *et al.*, 1996; Long and Barton, 1998; Byrne *et al.*, 2000; Christensen *et al.*, 2000), while others mark specific subregions within cotyledons, such as adaxial or abaxial sides (Lynn *et al.*, 1999; Siegfried *et al.*, 1999). Most genes that are expressed in cotyledon primordia are also expressed in leaf primordia in comparable patterns, supporting the hypothesis that cotyledons and leaves are homologous organs.

The *AINTEGUMENTA* (*ANT*) gene, which encodes a transcription factor that belongs to the AP2/ERBP family, is expressed in primordia of all shoot lateral organs, including cotyledons (Elliott *et al.*, 1996; Klucher *et al.*, 1996). Loss or gain of *ANT* function results in the decrease or increase in size of lateral organs, respectively. These changes in organ size are associated with changes in cell number, suggesting that *ANT* is required for growth of the shoot lateral organs by promoting cell division (Mizukami and Fischer, 2000). In embryos, *ANT* is expressed in the presumptive cotyledon as well as the boundaries between cotyledons from the late globular stage on, forming a ring-shaped pattern (Long and Barton, 1998). This expression pattern fits well with the view that cotyledon boundaries also have the potential to grow and give rise to cotyledons but their growth is suppressed by *CUC1* and *CUC2* in normal development (Aida *et al.*, 1997, 1999).

ASYMMETRIC LEAVES1 (*AS1*) is another regulator of shoot lateral organ formation (Byrne *et al.*, 2000). In a similar way to *ANT*, *AS1* expression in the embryo is detected as a ring that includes cotyledon primordia and the boundaries between cotyledons at least at the heart stage. *AS1* encodes a MYB transcription factor and is closely related to *PHANTASTICA* (*PHAN*) of *Antirrhinum* and *ROUGH SHEATH2* (*RS2*) of maize. All of these genes are shown to be negative regulators of knotted-like homeobox (*knox*) genes, which are important regulators of the SAM (see chapter 2 of this volume) (Schneeberger *et al.*, 1998; Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999; Byrne *et al.*, 2000; Ori *et al.*, 2000). *knox* genes are divided into two subclasses, class I and II.

with class I *knox* genes being expressed in the SAM and not in lateral organ primordia. Because misexpression of some of the class I *knox* genes disrupts patterning of the lateral organs, restriction of their expression is important to ensure proper differentiation of lateral organs (reviewed in Reiser *et al.*, 2000). In *as1* cotyledons, at least one class I *knox* gene, *KNAT1*, is ectopically expressed, suggesting that *AS1* negatively regulates *KNAT1* in cotyledons (Byrne *et al.*, 2000). At least two other class I *knox* genes, *KNAT2* and *STM*, are expressed in *Arabidopsis*. Expression analysis in *as1* leaves indicated that the mutation alters expression of *KNAT1* and *KNAT2*, but not *STM*. This result suggests that the restriction of *STM* may be regulated by a different mechanism when compared with the other two *knox* genes.

The *PINOID* (*PID*) gene, encoding a serine-threonine kinase, is also expressed in the presumptive cotyledons at the globular stage (Christensen *et al.*, 2000). Although the *pid* mutant displays only a minor phenotype in cotyledons, its inflorescence stems often fail to develop flowers, resulting in a pin-formed stem (Bennett *et al.*, 1995). This phenotype is reminiscent of that of the *pin1* mutant, which is defective in polar auxin transport, although young *pid* inflorescence stems show significant auxin transport activity (Okada *et al.*, 1991; Bennett *et al.*, 1995). Overexpression of *PID* results in altered sensitivity to auxin, suggesting that *PID* mediates auxin signalling (Christensen *et al.*, 2000). These findings suggest that the *PID* gene promotes formation of primordia in response to auxin.

Finally, *FILAMENTOUS FLOWER* (*FIL*), which encodes a putative transcription factor with a C2C2 zinc finger-like domain and a helix-loop-helix domain, is expressed in cells on the abaxial side of cotyledon primordia (Sawa *et al.*, 1999; Siegfried *et al.*, 1999). *FIL*, together with another redundant gene, *YABBY3*, specifies abaxial cell fate in cotyledons as well as other lateral shoot organs. Expression of *FIL* is first detected at the transition stage, suggesting that the molecular programme for specifying cell fates along the adaxial-abaxial axis of cotyledons starts soon after their initiation.

So far, we have little information on how the genes mentioned above are regulated and how they interact with one another. Which factors are responsible for initiation and maintenance of *ANT*, *AS1*, *PID* and *FIL*? Does their activation occur independently, or are there interactions between them? A problem when addressing such questions is that the corresponding mutants of these genes show no, or only slight, phenotypes in cotyledons. The lack of obvious phenotypes may be due, at least in part, to redundancy. For example, the *fil* mutant does not show an obvious phenotype related to adaxial-abaxial polarity unless it is combined with *yabby3* (Siegfried *et al.*, 1999). *ant* can cause complete loss of most floral organs when combined with *apetala2*, but the double mutant can still produce cotyledons and leaves (Elliott *et al.*, 1996). An extensive search for any redundant interactions should uncover the precise functions of these genes.

3.5.3 Pattern formation in the apical region

Overall, the expression patterns of the genes described above may imply that there are at least two kinds of positional information in the apical region of the embryo (figure 3.4C) (Aida *et al.*, 1999). One of these is the bilateral pattern, which consists of the medial region and the two equivalent lateral regions. This pattern is reflected by the stripe expression of the *STM* or *CUC* genes during the early stages of embryo development. By contrast, the ring-shaped expression of the *ANT* or *ASI* genes may reflect the other positional information, the radial pattern, which consists of the central and peripheral regions. This pattern is also reflected by late expression of *CUC* and *STM*, which are downregulated in the centre and periphery, respectively. Superimposition of these two patterns would establish three subregions consisting of the SAM, cotyledons and cotyledon boundaries.

It has been shown that alteration in polar auxin transport affects bilateral symmetry of the embryo, raising the possibility that auxin transport is involved in formation of the bilateral pattern. For example, embryos of *Brassica juncea* that are treated with inhibitors of auxin transport develop completely fused ring-shaped cotyledons, with no indication of bilateral symmetry (Liu *et al.*, 1993; Hadfi *et al.*, 1998). Furthermore, mutations in the *PIN1* gene, which is required for polar auxin transport, cause variable alterations in the number or position of cotyledons, as well as fusion of cotyledons (Okada *et al.*, 1991). Consistent with the cotyledon fusion phenotype, *pin1* embryos also show variable expression patterns of *CUC1* and *CUC2* (Aida and Tasaka, unpublished results). It is possible that *PIN1*-dependent polar auxin transport may establish an auxin distribution that reflects the bilateral pattern, and thereby defines the stripe expression of the *CUC* genes. In the root meristem of *Arabidopsis*, it has been proposed that an uneven auxin distribution that is dependent on transport of the hormone can direct tissue patterning (Sabatini *et al.*, 1999).

The ring-shaped cotyledons in embryos treated with inhibitors of auxin transport appear to reflect the fact that the radial pattern is still established in these embryos. This may suggest that auxin transport is not required for establishment of the radial pattern. Alternatively, the formation of the radial pattern may be less sensitive to alterations in auxin transport than the bilateral pattern.

3.6 Activation of genes involved in SAM maintenance

Once formed, the SAM continuously produces organ primordia in postembryonic development. Maintenance of the functional SAM requires coordination between proliferation of undifferentiated cells and loss of cells by differentiation. Genes involved in this process include *STM*, *WUSCHEL* (*WUS*) and *CLAVATA* (*CLV1*, *CLV2*, *CLV3*) of *Arabidopsis* (reviewed in Fletcher and

Meyerowitz, 2000; Waites and Simon, 2000; see also chapter 2 of this volume). Genetic and molecular analyses have suggested that the *WUS* and *CLV* genes interact with each other to maintain the stem cell population, while *STM* acts at a different level from *WUS* and *CLV* to promote the undifferentiated state and to prevent differentiation of the cells in the SAM. Expression of these genes starts at significantly early stages of embryogenesis, suggesting that the molecular programmes responsible for SAM maintenance become active before the morphological features of the SAM are fully apparent (figure 3.5).

3.6.1 Function of *STM* in SAM development

STM is a class I *knox* gene and its encoded protein is thought to function as a transcription factor (Long *et al.*, 1996). The *knox* genes are found in various plant species, including maize, rice, tobacco, tomato and *Arabidopsis* (Reiser *et al.*, 2000). Like *STM*, some members of the class I *knox* genes are expressed in the presumptive SAM of the early embryo (Smith *et al.*, 1995; Sato *et al.*, 1996); after the SAM has been established, their expression marks the undifferentiated region within the SAM in contrast to their exclusion from incipient lateral organ primordia. Misexpression of the *knox* genes in tobacco or *Arabidopsis* can induce ectopic SAMs on the adaxial surface of leaves (Sinha *et al.*, 1993; Chuck *et al.*, 1996). From these studies, the *knox* genes are thought to play a central role in the formation and maintenance of the SAM.

Phenotypes of *stm* mutants suggest that *STM* has a role in SAM development. In wild-type *Arabidopsis* embryos, three cell layers are formed at the presumptive SAM by the late torpedo stage. Subsequently, at the bent cotyledon stage, these cell layers broaden and adopt division patterns characteristic of the tunica and corpus, forming the SAM with a dome-shape that separates the base of cotyledons. In strong *stm* mutant embryos, cell division at the presumptive SAM is blocked at the torpedo stage and two cotyledons meet directly at the base, suggesting that *STM* promotes cell proliferation in the SAM (Barton and Poethig, 1993). In weak alleles of *stm*, on the other hand, a small number of densely staining meristematic cells are formed at the apex, but they are consumed to become leaf primordia soon after germination (Clark *et al.*, 1995; Endrizzi *et al.*, 1996). Together, these observations suggest that *STM* prevents meristem cells from being incorporated into organ primordia.

3.6.2 Activation and maintenance of *STM* expression

Several genes have been reported to affect *STM* expression during embryogenesis (Moussian *et al.*, 1998; Aida *et al.*, 1999; Lynn *et al.*, 1999). Among them, *CUC1* and *CUC2* are involved in activation of *STM* expression. Expression of the *CUC* genes begins slightly earlier than *STM* (figure 3.5). In addition, the *STM* RNA is not accumulated in the *cuc1 cuc2* double mutant, suggesting

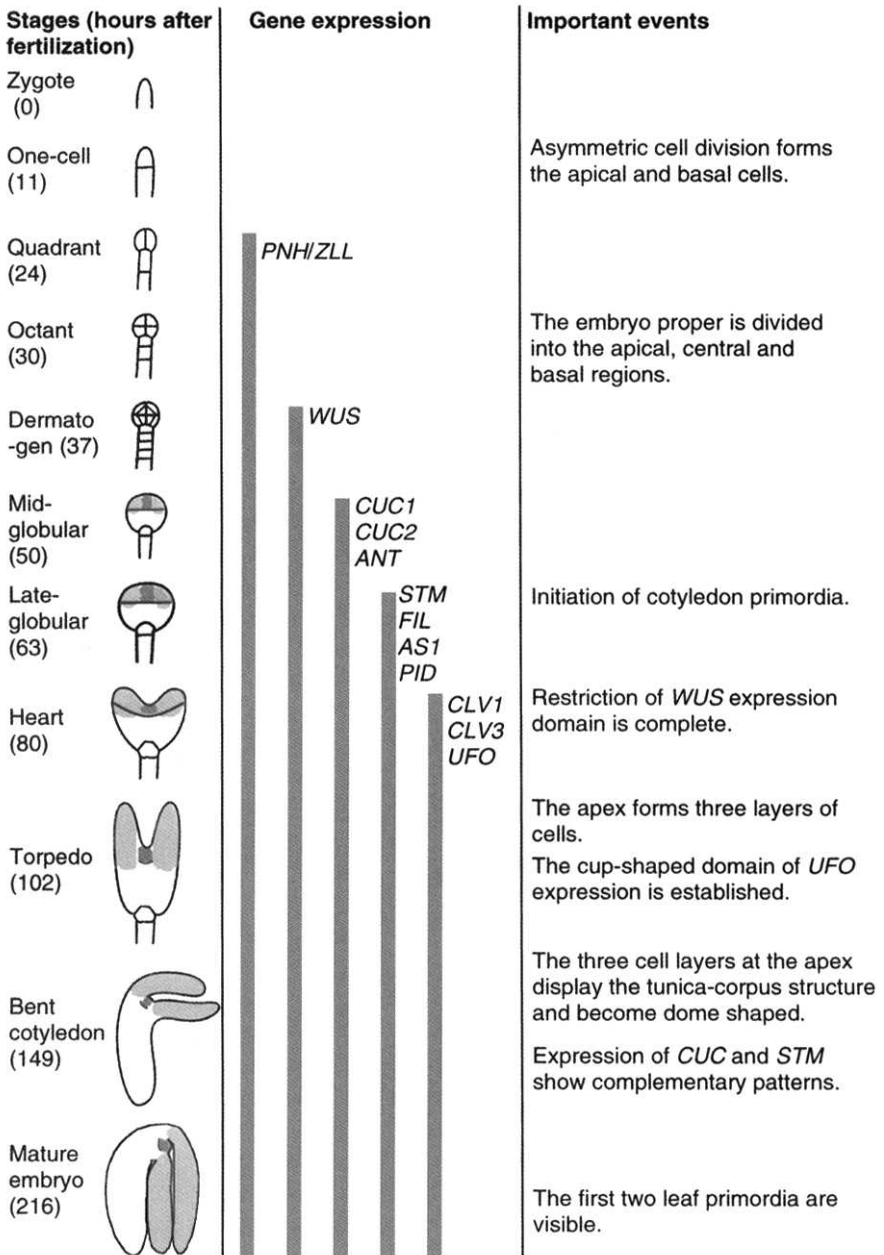


Figure 3.5 Shoot apical meristem (SAM) formation and expression of related genes. Stages and hours after fertilization for each stage are based on Jürgens and Mayer (1994). Abbreviations: *STM*, *SHOOT MERISTEMLESS*; *CUC*, *CUP-SHAPED COTYLEDONS*; *UFO*, *UNUSUAL FLORAL ORGANS*; *WUS*, *WUSCHEL*; *CLV*, *CLAVATA*; *PID*, *PINOID*; *AS1*, *ASYMMETRIC LEAVES1*; *FIL*, *FILAMENTOUS FLOWER*; *ANT*, *AINTEGUMENTA*; *PNH/ZLL*, *PINHEAD/ZWILLE*.

that expression of *STM* requires the activity of *CUC* genes (Aida *et al.*, 1999). Moreover, misexpression of *CUC1* under the control of CaMV 35S promoter is sufficient to induce ectopic expression of *STM*, leading to the formation of ectopic SAMs on the adaxial surface of cotyledons (Takada *et al.*, 2001). These results suggest that *CUC1* and *CUC2* are positive regulators of *STM* in SAM formation (figure 3.6).

How do the *CUC* genes promote *STM* expression? The N-terminal halves of proteins encoded by *CUC1* and *CUC2* share a highly conserved region called the NAC domain. NAC-domain containing proteins are only found in plants, including *Arabidopsis*, *Petunia*, rice and wheat, and constitute a large protein family (Xie *et al.*, 2000 and references therein). One of these proteins, NAC1 of *Arabidopsis*, was recently shown to function as a transcription activator. NAC1 consists of an N-terminal NAC domain that binds to DNA and a C-terminal activation domain, which is not strictly conserved among different NAC proteins (Xie *et al.*, 2000). Although neither the C-terminal half of *CUC1* or *CUC2* shows significant homology to NAC1, they are capable of activating transcription in yeast (Taoka and Tasaka, unpublished). In addition, a GFP-*CUC2* fusion protein localizes to the nucleus in *Arabidopsis* embryos, consistent with its proposed

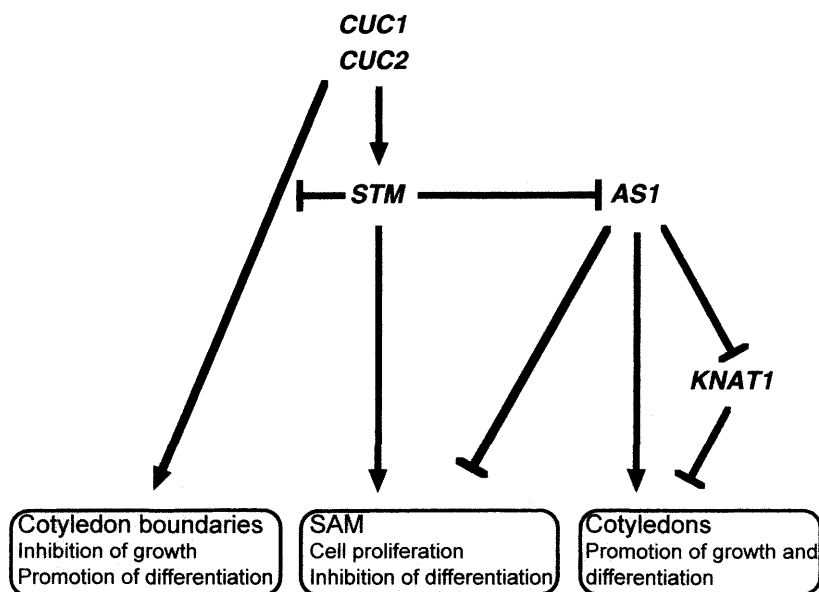


Figure 3.6 Interaction among genes involved in SAM formation. *CUC1* and *CUC2* activate *STM* expression for SAM formation. The *CUC* genes also specify the fate of the cotyledon boundary and their expression may be restricted by *STM* in later stages. *STM* negatively regulates *ASI* in the presumptive SAM. *ASI* promotes differentiation of cotyledons directly and/or through negatively regulating *KNAT1*. Without *STM* activity, *ASI* is ectopically expressed in the SAM and prevents its formation. Abbreviations: *CUC*, *CUP-SHAPED COTYLEDONS*; SAM, SHOOT APICAL MERISTEM; *STM*, SHOOT MERISTEMLESS; *ASI*, ASYMMETRIC LEAVES1.

function as a transcription factor (Aida and Tasaka, unpublished). Thus, *CUC* genes may either promote *STM* expression directly through activating its transcription or, alternatively, they may affect *STM* expression indirectly through activation of other regulatory genes.

By the bent cotyledon stage, expression of *CUC1* and *CUC2* is downregulated in the SAM, whereas *STM* is continuously expressed (Aida *et al.*, 1999). This observation suggests that once *STM* expression is established, *CUC1* and *CUC2* are not autonomously required for the maintenance of *STM* expression at the centre. Whether the downregulation of the *CUC* genes is required to maintain *STM* expression remains to be determined.

In *Petunia*, mutations in a NAC gene, *no apical meristem (nam)*, cause complete loss of the SAM and partial fusion of cotyledons as in the *cuc1 cuc2* mutant (Souer *et al.*, 1996). This finding suggests that the *nam* gene is required for SAM formation and cotyledon separation. The *nam* gene is likely to be a *Petunia* orthologue of *CUC2*, as is revealed by their sequence homology and mutant phenotype. Although the single mutation in the *nam* gene is sufficient to induce a detectable phenotype in *Petunia*, the mutant phenotype is still weaker than that of *cuc1 cuc2*, even in putative null alleles. For example, *nam* mutant seedlings frequently develop 'escape' SAMs after germination, while *cuc1 cuc2* mutants never develop SAMs unless they are induced artificially in tissue cultures (Souer *et al.*, 1996; Aida *et al.*, 1999). These observations suggest that *Petunia* may have a further gene that acts redundantly with the *nam* gene, and this gene can compensate for the loss of *nam* activity. Several NAC-like genes are found in *Petunia*, and some of these are indeed closely related to *nam* (Souer *et al.*, 1998).

Interestingly, *nam* expression is not detected at the presumptive SAM and is only detectable in the surrounding region. This observation suggests that the *nam* gene acts cell-non-autonomously in SAM formation. By contrast, *CUC1* and *CUC2* are detected at the presumptive SAM at least in the early developmental stages. This difference in expression patterns between the *nam* and *CUC* genes may reflect a difference in their mode of action. Alternatively, it is possible that the *CUC* genes could also function in a cell-non-autonomous manner.

Another gene that affects expression of *STM* during embryogenesis is *PINHEAD/ZWILLE (PNH/ZLL)*. Mutations in *PNH/ZLL* block development of the primary SAM shortly after its formation (McConnell and Barton, 1995; Moussian *et al.*, 1998). The primary SAM of the *pnh/zll* mutant frequently terminates before producing any leaves, forming a flat apex or a single leaf-like organ at the centre. After germination, however, most mutant seedlings develop escape SAMs from the axils of cotyledons. These secondary SAMs can continue to produce shoot organs and do not terminate, in contrast to the primary SAM. Thus, termination of the SAM occurs only during a limited stage of development. Consistent with this is the observation that *STM* expression in *pnh/zll* is only transiently altered at the bent cotyledon stage and is otherwise normal.

The termination of the SAM in *pnh/zll* is not fully penetrant: a considerable fraction of embryos does not show this phenotype. This could be attributed to another related gene, *ARGONOUTE1* (*AGO1*), which has partially overlapping functions with *PNH/ZLL* (Bohmert *et al.*, 1998; Lynn *et al.*, 1999). The *ago1* single mutant frequently shows termination of the primary SAM as in *pnh/zll*, and, in addition, it develops narrow cotyledons and leaves. On the other hand, the double mutant of *pnh/zll* and *ago1* develops highly abnormal embryos that grow very slowly, fail to develop cotyledons and do not accumulate the STM protein. Expression of *PNH/ZLL* is most prominent in the provascular cells, and is also detected at a weaker level at the presumptive SAM as well as the adaxial side of cotyledons. By contrast, *AGO1* is expressed ubiquitously throughout the embryo. These expression patterns, together with the phenotypes of the single and double mutants, suggest that the functions of these genes are pleiotropic and not restricted to SAM development (Lynn *et al.*, 1999).

In terms of the function of *PNH/ZLL* in primary SAM development, it is not clear why the mutant phenotype is restricted to the SAM that has just been formed. Since the SAM at this stage contains only about 70–110 cells, of which the incipient primordia of the first leaves constitute a significant portion (Medford *et al.*, 1994), it is possible that the SAM is labile and particularly sensitive to the loss of *PNH/ZLL* activity.

PNH/ZLL and *AGO1* encode proteins related to rabbit eIF2C, which has been proposed to be a part of a protein complex that can stimulate the initiation of translation (Zou *et al.*, 1998; Lynn *et al.*, 1999). Proteins related to *PNH/ZLL* are found in various eukaryotes, including fungi, *Caenorhabditis elegans*, *Drosophila* and human. Among them, QDE-2 of *Neurospora* and RDE-1 of *C. elegans* are required for quelling and RNA interference, respectively (Tabara *et al.*, 1999; Catalanotto *et al.*, 2000). Both phenomena are thought to be comparable to post-transcriptional gene silencing (PTGS) in plants (Bosher and Labouesse, 2000). Another protein of *Drosophila*, STING/AUBERGINE, is required to enhance translation of a specific developmental gene, *oskar* (Willson *et al.*, 1996; Schmidt *et al.*, 1999). These findings may suggest that *PNH/ZLL* and *AGO1* are involved in regulating the expression of other developmental genes, including *STM*, at a post-transcriptional level, possibly by interacting with RNA. Recently, it has been shown that the *ago1* mutant is also defective in PTGS, providing a link between PTGS and the regulation of developmental genes. It is not known whether *pnh/zll* is also defective in PTGS (Fagard *et al.*, 2000).

Mutations that affect expression of a class I *knox* gene have also been reported in rice. Expression of the rice class I *knox* gene, *OSH1*, is already observed at the globular stage in the presumptive SAM (Sato *et al.*, 1996). Recessive mutations in four genes, *SHOOTLESS1–4* (*SHL1–4*), block formation of the SAM (Sato *et al.*, 1999). The normal rice embryo develops three embryo specific appendages, the coleoptile, epiblast and scutellum, whose origins have been controversial. In all *shl* mutants, the coleoptile and epiblast are lost while

the scutellum remains intact. These findings suggest that the differentiation of coleoptile, epiblast and SAM share a common mechanism, while differentiation of the scutellum is regulated independently. Expression of *OSH1* is significantly reduced in *shl1* and *shl2* embryos but not in *shl3* and *shl4*, suggesting that *SHL1* and *SHL2* acts upstream of *OSH1* to promote its expression.

3.6.3 Genes regulated by STM

STM encodes a homeodomain protein and is thought to act as a transcription factor. Although no direct target of *STM* has been shown, there are some candidates whose expression is dependent on *STM* activity. One such gene is *UFO*, the expression of which starts at the early heart stage within the domain of *STM* expression (Long and Barton, 1998). No *UFO* signal is detected in *stm* mutant embryos, suggesting that the expression of *UFO* requires *STM*. The function of *UFO* during embryogenesis, however, remains to be determined.

UFO was originally identified as a gene involved in floral development, as mutations in *UFO* disrupt patterning of floral organs (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). *UFO* encodes an F-box protein that can interact with the *Arabidopsis* SKP1-like proteins, *ASK1* and *ASK2*, and is thought to be involved in targeting specific proteins for ubiquitin-mediated degradation (Ingram *et al.*, 1995; Samach *et al.*, 1999). In early floral meristems, *UFO* is expressed as a cup-shaped domain that may include presumptive whorls 2 and 3. It is thought to activate expression of the B class of homeotic gene, *APETALA3* (*AP3*), in a cooperative manner with the floral transcription activator, *LEAFY* (*LFY*) (Lee *et al.*, 1997; Parcy *et al.*, 1998). Because *LFY* expression is ubiquitous in floral meristems at the time of *AP3* induction, it is proposed that spatially restricted expression of *UFO* is important in defining the expression domain of *AP3* (see chapter 4 of this volume for a more detailed discussion). Strikingly, the cup-shaped pattern of *UFO* expression is already apparent in the late torpedo stage embryo, suggesting that a prepattern for the floral meristem is established, at least in part, during embryogenesis and is somehow maintained throughout the vegetative and reproductive SAMs (Long and Barton, 1998; Scheres, 1998). The nature of the prepattern that defines *UFO* expression, however, is totally unknown.

Another candidate target of *STM* is *ASI*, which is involved in cotyledon formation (Byrne *et al.*, 2000). During embryogenesis, *ASI* is expressed at the cotyledon primordia and is repressed at the presumptive SAM. In *stm* embryos, however, *ASI* is derepressed in this region, suggesting that *STM* negatively regulates *ASI* (figure 3.6). In addition, the *as1* mutation suppresses strong *stm* mutant phenotypes and largely recovers leaf production. These results suggest that, in *stm* embryos, ectopic expression of *ASI* at the presumptive SAM blocks proliferation of meristem cells and promotes cells being incorporated into organ primordia. Although *STM* can repress *ASI* expression at the presumptive SAM,

expression of these genes overlaps at the cotyledon boundaries of the wild-type heart stage embryo (Byrne *et al.*, 2000). This observation indicates that there may be other factors that prevent negative regulation of *AS1* by *STM* at the cotyledon boundaries.

3.6.4 *Activation of genes involved in stem cell specification and maintenance*

At the summit of the SAM, a small number of pluripotent stem cells are formed. These stem cells divide slowly to generate daughter cells that are incorporated into differentiating organ primordia, and thereby serve as an ultimate source of all shoot organs. The stem cell population in the SAM is maintained by the interaction of two classes of key regulators, *WUS* and *CLV* (reviewed in Fletcher and Meyerowitz, 2000; Waite and Simon, 2000; see also chapter 2 of this volume).

In *wus* mutant embryos, cells at the base of cotyledons proliferate to some extent and form a flat structure instead of the dome-shaped SAM (Laux *et al.*, 1996). This structure has significantly low activities of cell proliferation and organ formation, and terminates after having produced only a few leaves. Based on this phenotype, it is proposed that *WUS* is required for specification of stem cells within the SAM. *WUS* encodes a homeodomain-containing protein that belongs to a different class from the KNOX family (Mayer *et al.*, 1998), which is expressed from the dermatogen (16-cell) stage embryo onwards. In the active SAM, *WUS* is expressed in a small group of cells underneath the presumed stem cell population, suggesting that *WUS* could affect the fate of the stem cells in a non-cell-autonomous fashion.

Loss of *CLV1*, *CLV2* or *CLV3* activities causes an accumulation of undifferentiated cells in the shoot apex without affecting the rate of cell proliferation, suggesting that the *CLV* genes restrict stem cell number in the SAM (Clark *et al.*, 1993, 1995; Kaye and Clark, 1998; Laufs *et al.*, 1998). Genetic and biochemical analyses indicate that the three *CLV* genes encode components of a signalling pathway, in which the *CLV1* and *CLV2* proteins are components of a receptor kinase complex and *CLV3* is a ligand that activates the complex (Clark *et al.*, 1995, 1997; Fletcher *et al.*, 1999; Jeong *et al.*, 1999; Trotochaud *et al.*, 2000). *CLV1* and *CLV3* begin to express at the apex of the heart stage embryo (Long and Barton, 1998; Fletcher *et al.*, 1999), but a detailed expression pattern of *CLV2* has not yet been determined (see chapter 2 of this volume).

Studies on genetic interaction between the *WUS* and *CLV* genes have revealed a model for stem cell maintenance in the SAM (figure 3.7A) (Brand *et al.*, 2000; Schoof *et al.*, 2000). In this model, the *WUS*-expressing organizing centre produces a signal that maintains stem cell identity of the overlying cells. The stem cells in turn signal back to the organizing centre to restrict the *WUS*-expression region through the signalling pathway mediated by the *CLV* genes.

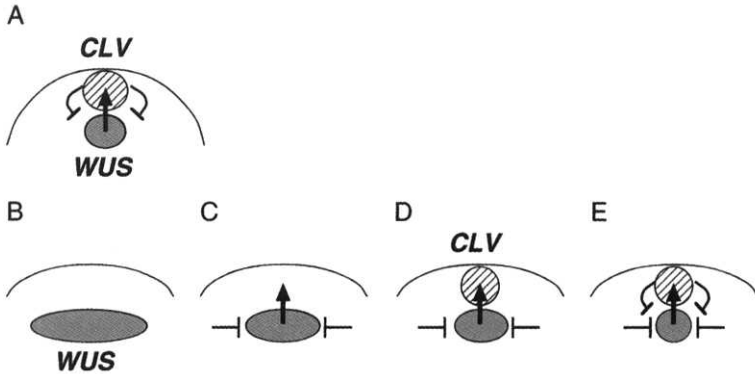


Figure 3.7 The initiation and maintenance of stem cells in *Arabidopsis* shoot apex. A: A model for stem cell maintenance in the active SAM (adopted from Schoof *et al.*, 2000). B–E: A model for initiation of the *WUS*-*CLV* feedback loop during embryogenesis. Firstly, *WUS* expression starts in the four apical inner cells at the 16-cell stage and specifies the organizing centre (B). Subsequently, a *WUS*-dependent signal induces the stem cell fate of the overlying cells (C). These overlying cells are specified to become stem cells and start to express the *CLV* genes (D). The stem cells then begin to signal back to restrict the underlying *WUS* expression region (E). The restriction of *WUS* region is also promoted by *CLV*-independent factors that are already active before the onset of the *CLV* genes (C–E). For simplification, embryos are depicted in the same size and shape. Abbreviations: SAM, shoot apical meristem; *WUS*, *WUSCHEL*; *CLV*, *CLAVATA*.

The interaction between *WUS* and *CLV* could thus establish a feedback loop between the stem cells and the underlying organizing centre.

How is this feedback loop initiated? Expression studies indicate that *WUS* starts to express earlier than *CLV1* and *CLV3* (figure 3.5). Because misexpression of *WUS* is sufficient to induce ectopic stem cells, as indicated by ectopic expression of the stem cell marker *CLV3* (Schoof *et al.*, 2000), it is possible that the initiation of *WUS* alone could induce the feedback loop (figure 3.7B–E). It remains to be determined, however, whether the initiation of *CLV* expression is dependent solely on *WUS* activity or is regulated by other factors that promote SAM formation, such as *CUC1*, *CUC2* or *STM*. What is known from analysis of *CLV1* expression in *stm* embryos is that the initiation of *CLV1* does not require *STM* (Long and Barton, 1998).

WUS starts to express in the four apical inner cells of the 16-cell stage embryo. Subsequently, expression continues in a subset of the cells derived from these four cells and finally, by the heart stage, expression becomes restricted to the cells underneath the presumed stem cells (Mayer *et al.*, 1998). This restriction is delayed in embryos of all three *clv* mutants, suggesting that the *CLV* genes negatively regulate *WUS* expression (Schoof *et al.*, 2000). However, downregulation of *WUS* still occurs in all *clv* embryos, indicating that not only the *CLV* genes but also other factors are involved in the restriction of *WUS* expression. These factors must be active before the onset of *CLV* gene

expression, because the restriction already takes place immediately after the initiation of *WUS* expression. In *fasciata1* (*fas1*) and *fas2* mutant seedlings, the organization of the SAM is variably disrupted (Kaya *et al.*, 2001). This phenotype is associated with variably expanded expression of *WUS*, suggesting that the *FAS* genes are required for stable restriction of *WUS*, at least at the seedling stage. Detailed phenotypic analyses as well as double mutant analysis suggest that the *FAS* and *CLV* genes act in different pathways (Leyser and Furner, 1992). *FAS1* and *FAS2* encode subunits of the *Arabidopsis* counterpart of chromatin assembly factor-1 (CAF1), which is thought to be involved in chromatin assembly during DNA replication and repair (Verreault, 2000; Kaya *et al.*, 2001). It is proposed that the *FAS* genes promote stable maintenance of an epigenetic state for gene expression. Although expression of the *FAS* genes and their effect on *WUS* expression is not observed during early embryogenesis, it is possible that *FAS*-mediated chromatin assembly is involved in stable restriction of *WUS* in early stage embryos.

If *WUS* is sufficient to form stem cells and to initiate the feedback loop between the stem cells and the organizing centre, the activation of *WUS* expression should be a key step for stem cell formation. What factors regulate this step? *WUS* mRNA is detected in *stm* as well as *cuc1 cuc2* embryos (Mayer *et al.*, 1998; Aida and Tasaka, unpublished), suggesting that the activation of *WUS* does not require any of the *STM*, *CUC1* or *CUC2* genes. These results, however, might be trivial because *WUS* expression starts earlier than that of the other three genes (figure 3.5). Of the genes reported to be involved in SAM formation, only *PNH/ZLL* expression begins earlier than *WUS* (figure 3.5) (Lynn *et al.*, 1999). *PNH/ZLL* mRNA is detected in all cells in the embryo proper at the quadrant stage and *WUS*-expressing cells arise within the area of *PNH/ZLL* expression. In addition, *pnh/zll* embryos occasionally develop a flat apex, similar to *wus* (Moussian *et al.*, 1998). Therefore, *PNH/ZLL* could be a candidate gene for the regulation of *WUS* activation. Functional redundancy and pleiotropy of *PNH/ZLL*, however, might make it difficult to test this hypothesis.

3.7 Concluding remarks

With the identification of SAM development mutants and subsequent cloning and functional analyses of the corresponding genes over the past five years, we now have a much better understanding of how the SAM is formed during embryogenesis. Embryonic expression of the genes involved in maintenance of the SAM, such as *STM*, *WUS*, and *CLV*, have revealed that SAM-specific programmes are activated significantly earlier than previously thought. In addition, possible upstream regulators of *STM* or its related *knox* genes have been identified, such as *CUC1*, *CUC2*, *PNH/ZLL* and *AS1*. Further analyses of the regulation and function of these genes should provide a link between activation of

the SAM programmes and earlier patterning processes in the embryo. Analyses of the function of the *GNOM* gene and its related cellular process have advanced our understanding of the establishment of polarity, which is probably one of the earliest and most fundamental processes in plant development. The next challenge should be further detailed analyses of the genes mentioned above, as well as identification of new genes that link the establishment of apical-basal polarity with activators of SAM-specific programmes.

With many *Arabidopsis* genes involved in SAM initiation already identified, we can now look at diversity of meristem initiation processes in different species at the molecular level. As mentioned in section 3.2, the times at which morphological features of the SAM become apparent varies among species. It would be interesting to test whether these differences are reflected in differences in expression of SAM-specific genes. For example, the counterparts of the *WUS* and *CLV* genes may be expressed earlier in species such as *Downingia* or *Stellaria*, which develop distinct cytological zonation at the globular stage (Pritchard, 1964; Kaplan and Cooke, 1997).

Some of the genes involved in SAM formation are plant specific and have not so far been found in animals or fungi; examples are *CUC1*, *CUC2* and *nam* of the NAC family. *Arabidopsis* has more than a hundred members in this family (Riechmann *et al.*, 2000; The Arabidopsis Genome Initiative, 2000), and the functions of members in the family are significantly diverged. Analyses of the *CUC* and *nam* genes reveal that part of the SAM initiation process is regulated by a common genetic mechanism in two dicotyledonous species, *Arabidopsis* and *Petunia* (Souer *et al.*, 1996; Aida *et al.*, 1997). Extensive research on genes of this class in other angiosperms, or species of other taxa, should yield insights into how plants have adopted these gene products for SAM formation, a unique developmental process of plants.

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4 Genetic control of reproductive meristems

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and Hong Ma

4.1 Introduction: scope and emphasis

In higher plants, postembryonic development depends on the activity of meristems that consist of groups of undifferentiated cells whose distinctive pattern of division contributes to the growth of the plant. Generally speaking, cells in the meristem divide to maintain the meristem and also to give rise to new structures. The shoot apical meristem (SAM), formed during embryogenesis, is the ultimate source for all postembryonic aerial structures of flowering plants, including stems, leaves, flowers and fruits. This chapter focuses on the activity of so called 'reproductive meristems' that are responsible for producing the reproductive parts of the plant, including inflorescences and flowers. There is a brief description of different types of reproductive meristems and their characteristics, followed by a discussion of our current understanding of the genetic control of meristem formation and function, including the genetic network that regulates the timing of reproductive development, the genes that specify the type of reproductive meristems, the genes that control the size of reproductive meristems, and the interactions between different sets of regulatory genes.

Several recent reviews have discussed specific aspects of reproductive meristems. They are referred to throughout this chapter and the reader is encouraged to seek them out for further information. Rapid progress has been made in the understanding of gene functions controlling various aspects of reproductive meristems, even in the just last two or three years. Given that the majority of this work has been performed with *Arabidopsis thaliana*, this chapter emphasises this species and, therefore, if the plant of a particular gene is not specified, it is *Arabidopsis*. Nevertheless, relevant studies in others plants are included, particularly *Antirrhinum majus*, *Petunia*, tomato, and maize.

Most of the mutant analyses cited here involve recessive loss-of-function mutations, which test whether a gene is required for a particular process. On the other hand, approaches to constitutively express or overexpress a cloned gene (gain-of-function experiments) can probe gene functions by testing their sufficiency for a specific aspect of flower development. This approach has the advantage over the use of dominant mutations, where it is not always known whether the dominance results from a gain in gene function versus

haplo-insufficiency. Furthermore, in this chapter several instances where gain of gene function is controlled by an inducible system are also discussed. It can be seen that the interpretation of the results and genetic interactions depends on the experimental approach used.

4.2 Functions and properties of reproductive meristems

In flowering plants, such as *Arabidopsis thaliana* and *Zea mays* (maize), the SAM undergoes several distinct transitions in identity during the life of the plant (Steeves and Sussex, 1989; Irish and Nelson, 1991; Veit *et al.*, 1993; Pidkowich *et al.*, 1999). A major transition, the switch from vegetative growth to reproductive growth, is regulated by both developmental signals and environmental cues (Simpson *et al.*, 1999). In some plants, the vegetative meristem is converted to a single floral meristem following flowering transition, resulting in a single terminal flower at the apex of a branch (figure 4.1A, e.g. *Magnolia liliflora*) (Coen, 1991; Ma *et al.*, 1998). In many plants, however, several to many flowers form a reproductive shoot called an inflorescence and flowering transition results in the conversion of a vegetative meristem to an inflorescence meristem. The inflorescence meristem then produces floral meristems directly (e.g. in *Arabidopsis*), or intermediate meristems (e.g. in maize) that give rise to floral meristems. Finally, the floral meristem generates floral organs.

Inflorescence meristems that are responsible for the architecture of the inflorescences can be grouped into two basic types, determinate and indeterminate. A large number of plants have indeterminate inflorescences (figure 4.1B, e.g. *Arabidopsis thaliana* and *Antirrhinum majus*), whose terminal meristem is maintained until the plant ceases growth (Coen and Meyerowitz, 1991). Many other plants produce determinate inflorescences with a limited number of flowers, including terminal flower(s) (figure 4.1C, e.g. *Dianthus chinensis* with a 'dichasium' inflorescence) (Coen, 1991; Ma *et al.*, 1998). Both indeterminate and determinate inflorescences can be further subdivided according to their architectures (figure 4.1). For example, the indeterminate inflorescences found in *Arabidopsis* and *Antirrhinum* are of the 'raceme' type, with an unbranched, elongated main axis with flowers having relatively similar pedicel lengths (figure 4.1B). By contrast, 'corymbs' (figure 4.1D, found in apple; Ma *et al.*, 1998), though also having an unbranched, elongated main axis, have reducing pedicel lengths from the base to the apex, resulting in a flat-topped appearance. Other types include the 'umbel', or umbrella-shaped, found in carrot and onions (figure 4.1E), the 'capitulum' or head in sunflower and daisy (figure 4.1F), and the 'hypanthodium' of figs with internal flowers surrounded by an enlarged receptacle (figure 4.1G).

Variations of the determinate inflorescences are also found; for instance, dichasium is a specific case of the common 'cyme' inflorescence, which produce

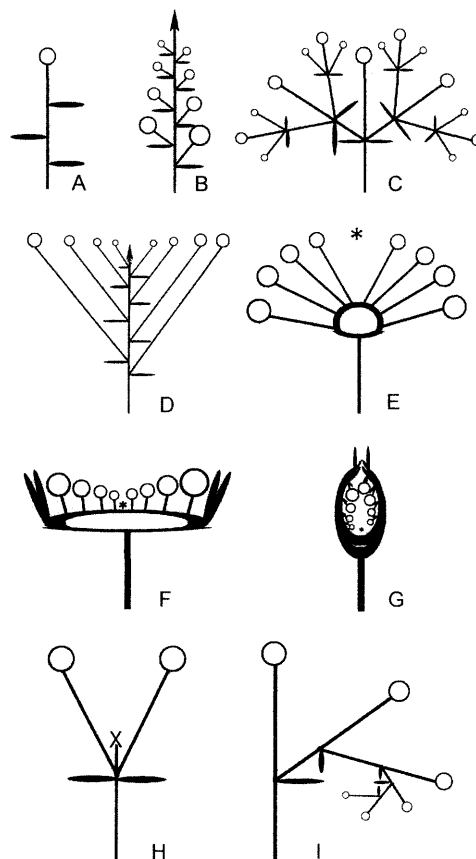


Figure 4.1 Schematic representation of inflorescence architectures. A: Single terminal flower. B: Raceme, an indeterminate inflorescence. C: Cyme or dichasium, a determinate inflorescence. D: Corymb. E: Umbel. F: Capitulum (head). G: Hypanthodium. H: Dichasium lacking the apical flower. I: Cincinnus.

a terminal flower and two or more branches from below the terminal flower. In *Lonicera japonica* (figure 4.1H, Japanese honeysuckle; Ma *et al.*, 1998), the top flower of a dichasium inflorescence is not developed. Another example is *Iris tectorium* (figure 4.1I), which has a 'cincinnus' inflorescence that lacks the flower on one side at each level of the branched structure. Some plants develop an overall indeterminate inflorescence that has determinate branches, such as those in members of the Lamiaceae family (mint). These various types of inflorescence architecture are produced from different inflorescence meristems. The morphology of the inflorescence meristem is an important contributing factor to the structure of the inflorescence and can vary from cup-like to flat (e.g. in sunflower; Esau, 1977), and from mound-shaped (e.g. *Arabidopsis*) to

finger-shaped (e.g. in maize; Veit *et al.*, 1993; McSteen *et al.*, 2000). Moreover, the relative positions of floral meristems on an inflorescence meristem also contribute to the overall inflorescence structure.

In many species, particularly annuals and biennials, the transition of SAM from a vegetative meristem to an inflorescence meristem marks the end of vegetative growth and the beginning of reproductive development (figure 4.2A). The replacement of the vegetative meristem by the inflorescence meristem terminates further vegetative development (figure 4.2A). Plants with this kind of vegetative-inflorescence transition flower only once during their entire life cycle. In other species, such as in many perennials, reproductive development occurs in certain portions of the shoot, but vegetative growth of the plant is not terminated (Howell, 1998). There are two major kinds of vegetative-to-reproductive transition patterns in these plants. In the first, the inflorescence meristem does not terminate the vegetative meristem; the two types of meristem alternate and produce separate and consecutive regions of the shoot (figure 4.2B, C). In the second, the inflorescence meristem terminates the main vegetative meristem, but new vegetative meristem(s) can arise from lateral positions (figure 4.2D). Grasses and other perennial herbaceous plants can generate new shoots from basal regions of the plant and terminate shoot development with reproduction. Thus they can be considered as special cases of the latter type.

The function of the floral meristem is to produce floral organs. The flower of *Arabidopsis* has four whorls of organs: sepal, petal, stamen and carpel.

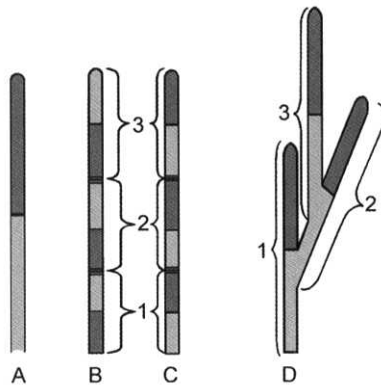


Figure 4.2 Three types of vegetative-inflorescence patterns. A: Simple vegetative-inflorescence. B: Consecutive inflorescence-vegetative development. C: Consecutive vegetative-inflorescence development. 1, 2 and 3 represent different cycles, in perennial plants, generally one cycle per year. D: Inflorescence development terminates the shoot (1); secondary shoot develops from the vegetative portion and again terminates in an inflorescence (2); this can continue repeatedly (3). In perennials, there is often one shoot cycle per year. Key: ■, inflorescence stem; □, vegetative stem.

Floral organ primordia develop into concentric whorls on the floral meristem, starting from the outer whorl. Sepal primordia arise first in the outer whorl at the periphery of the meristem dome. Petal primordia appear interior to the sepals. Further to the interior are stamen primordia and, finally, carpel primordia occupy the center of the floral primordium (figure 4.3). Different species can have different number of whorls and different number of organs in each whorl. In basal angiosperms and some monocotyledonous plants, such as tulip, the sterile outer organs are not easily differentiated into sepals and petals and are instead called tepals (Bowman, 1997). Furthermore, floral organs in flowers of basal angiosperms often have a spiral phyllotaxy rather than the whorled phyllotaxy found in higher eudicotyledonous and monocotyledonous plants. Therefore, floral meristems have diverged to generate a variety of floral forms.

In grasses, the inflorescence is called a spike (wheat or barley), a panicle (oat and rice), or ear (female in maize) and tassel (male in maize). The flowers are referred to as florets, and one to many florets can be found in a spikelet, or miniature branch; in maize a spikelet has two florets (Clifford, 1987; Veit *et al.*, 1993; McSteen *et al.*, 2000). Therefore, the inflorescence meristem produces spikelet meristems, each of which gives rise to two floret meristems. In the maize tassel, the inflorescence has a main spike and a number of branch spikes, whereas the normal ear has a single spike. The grass spikelets and florets possess highly specialized organs. Each spikelet has a pair of glumes, which are bract-like organs. Interior to the glumes, the sterile organs of the florets are called lemma, palea and lodicules. Although the evolutionary relationship between the grass sterile floral organs and the perianth of eudicotyledonous plants is not clear, molecular genetic evidence supports homologies between lemma/palea and sepals, and between lodicules and petals (Ambrose *et al.*, 2000; Ma and dePamphilis, 2000).

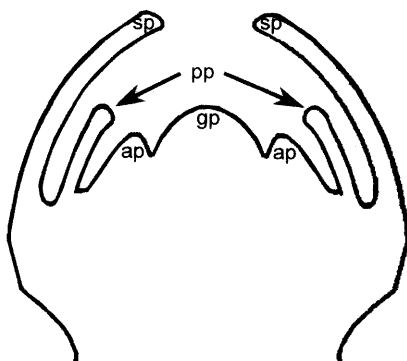


Figure 4.3 Young flower bud showing the position of primordia of floral organs. Abbreviations: sp, sepal primordia; pp, petal primordia; ap, stamen primordia; gp, gynoecial primordia.

4.3 Control of flowering time

Reproductive meristems are formed as a consequence of the flowering transition (Bernier, 1988; Bernier *et al.*, 1993). The decision to undergo this critical transition from vegetative to reproductive development represents a major commitment of resource distribution by the plant. The success of this decision depends heavily on the environmental conditions, such as light, temperature, nutrient supply and availability of water. Therefore, these environmental factors play critical roles in controlling flowering time. At the same time, given a particular set of conditions, the plant needs to achieve sufficient vegetative growth to be able to support the costly process of reproduction. This means that plants must also regulate the flowering process in order to delay it until there has been enough vegetative growth.

Genetic studies in *Arabidopsis* have uncovered a large number of genes that affect flowering time (table 4.1 and figure 4.4). *Arabidopsis* is a facultative long-day plant and therefore flowers earlier under long days than short days. Under long days, *Arabidopsis* flowers after producing several rosette leaves. Plants of late-flowering mutants and ecotypes flower after producing dozens of rosette leaves, whereas early flowering mutants flower following the production of only a few leaves, or, in some cases, none at all. This chapter focuses on the genes that regulate flowering but does not describe the phenotypes in detail.

Genetic and physiological studies have placed many of the genes that affect flowering into distinct pathways (Koornneef *et al.*, 1991; Martinez-Zapater *et al.*, 1994; Simpson *et al.*, 1999). Light is the most important environmental signal regulating *Arabidopsis* flowering. Several genes were discovered because mutations in them cause late flowering under long days but generally do not affect flowering under short days. These genes include *CO*, *FD*, *FE*, *FHA*, *FT* and *GI* and are thought to be components of the long-day pathway. Another group of genes, *FCA*, *FY*, *FPA*, *FVE*, *LD* and *FLD*, promote flowering under both long days and short days and are part of the autonomous pathway. Many other genes have been identified more recently (Simpson *et al.*, 1999).

Like many other plants, *Arabidopsis* flowering is also accelerated by an extended cold treatment called vernalization, which is similar to a winter season. Because vernalization can suppress the effect of mutations in genes of the autonomous pathway and is independent of photoperiods, it is thought to operate through a separate pathway. Mutational analyses have uncovered the *VRN1* and *VRN2* genes that may be involved in the vernalization pathway (Chandler *et al.*, 1996).

In recent years, rapid progress has been made in the understanding of many gene functions, particularly those in the long-day and autonomous pathways. Furthermore, new insights have been obtained on the mechanisms of the flowering time genes, at least part of which involve the control of reproductive meristem formation and the regulation of relevant meristem genes.

Table 4.1 *Arabidopsis* genes affecting flowering time

Gene	Mutant/ecotype phenotypes ^a	Protein functions
<i>ABA</i>	early flowering under SD, ABA deficient	ABA synthesis
<i>ABI1</i>	early flowering under SD, ABA insensitive	protein phosphatase
<i>ABI2</i>	early flowering in <i>fca</i> mutant, ABA insensitive	protein phosphatase
<i>AGL20/SOC1</i>	late flowering	MADS-box protein
<i>CCA1</i>	defective circadian clock	MYB-domain protein
<i>CO</i>	late flowering under LD	transcription factor
<i>CRY1</i>	defective circadian clock	blue-light receptor
<i>DET2</i>	late flowering	brassinolide synthesis
<i>EAF1</i>	early flowering, resistant to GA biosynthesis inhibitor	
<i>EFS</i>	early flowering in <i>fwa</i> mutant	
<i>ELF1</i>	early flowering	
<i>ELF2</i>	early flowering	
<i>ELF3</i>	early flowering, long hypocotyl, defective circadian clock	
<i>EMF1</i>	early flowering, upon germination	
<i>EMF2</i>	early flowering, upon germination	
<i>EIN2</i>	late flowering, ethylene insensitive	
<i>ESD1</i>	early flowering under SD	
<i>ESD2</i>	early flowering under SD	
<i>ESD3</i>	early flowering under SD	
<i>ESD4</i>	early flowering under SD	
<i>ETR1</i>	late flowering, ethylene insensitive	ethylene receptor
<i>FCA</i>	late flowering	RNA-binding protein
<i>FD</i>	late flowering	
<i>FE</i>	late flowering	
<i>FHA/CYR2</i>	late flowering	blue-light receptor
<i>FLC/FLF</i>	dominant allele causing late flowering	MADS-box
<i>FLD</i>	null mutant early flowering	
<i>FKF1</i>	defective circadian clock and flowering time	LOV, F-box, kelch repeats
<i>FPA</i>	late flowering	
<i>FPF1</i>	overexpression results in early flowering	novel protein
<i>FRI</i>	late flowering	
<i>FT</i>	late flowering	homolog of TFL1/CEN
<i>FVE</i>	late flowering	
<i>FWA</i>	late flowering	homeodomain protein
<i>FY</i>	late flowering	
<i>GA1</i>	late flowering under SD, GA deficient	GA synthesis
<i>GAI</i>	late flowering under SD, GA insensitive	putative transcription factor
<i>GI</i>	late flowering	putative transmembrane protein
<i>HY1</i>	early flowering, long hypocotyl	phytochrome chromophore synthesis

Table 4.1 (continued)

Gene	Mutant/ecotype phenotypes ^a	Protein functions
<i>HY2</i>	early flowering, long hypocotyl	phytochrome chromophore synthesis
<i>LD</i>	late flowering	homeodomain transcription factor
<i>LHY</i>	dominant defective circadian clock	MYB-domain protein
<i>PHYA/HY8</i>	reduced flowering promotion, long hypocotyl under far-red light	red/far-red light receptor
<i>PHYB/HY3</i>	early flowering, long hypocotyl	red light receptor
<i>PHYD</i>	enhancing early flowering of <i>phyB</i> mutant	red light receptor
<i>PHYE</i>	early flowering in <i>phyA phyB</i> double mutant	red light receptor
<i>RGA</i>		putative transcription factor
<i>SPL3</i>	constant expression causes early flowering	transcription factor
<i>SPY</i>	early flowering, slender, GA signaling	tetratricopeptide repeat protein
<i>SVP</i>	early flowering	MADS-box; transcription factor
<i>TFL1</i>	early flowering	phosphatidylethanolamine-binding protein, CEN homologue
<i>TFL2</i>	early flowering	
<i>ZTL</i>	defective circadian clock and flowering time	LOV, F-box, kelch repeats

^aUnless otherwise indicated, the phenotypes are due to recessive mutations. Abbreviations: SD, short day; LD, long day; ABA, abscisic acid; GA, gibberellic acid; LOV, light, oxygen or voltage.

4.3.1 Genes that inhibit premature reproductive development

To achieve maximum reproductive success, plants must ensure that sufficient vegetative development has occurred before switching to reproduction, while taking into account available resources and growth conditions. Therefore, it is reasonable to propose that there are genetic programs that actively prevent precocious reproductive development. Indeed, mutant analysis has uncovered genes that are required for this delay, called *EMF1* and *EMF2* (Sung *et al.*, 1992; Yang *et al.*, 1995; Haung and Yang, 1998; Chou and Yang, 1999). The *emf1* and *emf2* mutants can produce a flower immediately following seed germination without the production of any leaves. A number of genetic and molecular studies support the hypothesis that various flowering promotion pathways can overcome the repressive effects of the *EMF* genes. In addition, several others genes also contribute to extending vegetative development and delaying reproductive development, including *ELF* and *TFL* genes (Simpson *et al.*, 1999).

4.3.2 Photoreceptors, the circadian clock and the long-day pathway

The ability to perceive and process the day-length signal ultimately depends on light receptors and the circadian clock. In *Arabidopsis*, there are five genes encoding the red/far-red receptor phytochrome: *PHYA* to *PHYE* (Sharrock and Quail, 1989; Clack *et al.*, 1994). *PHYA* is light-labile and primarily a far-red light receptor; *phyA* mutations (also called *hy8* and *fhy1*; Parks and Quail, 1993;

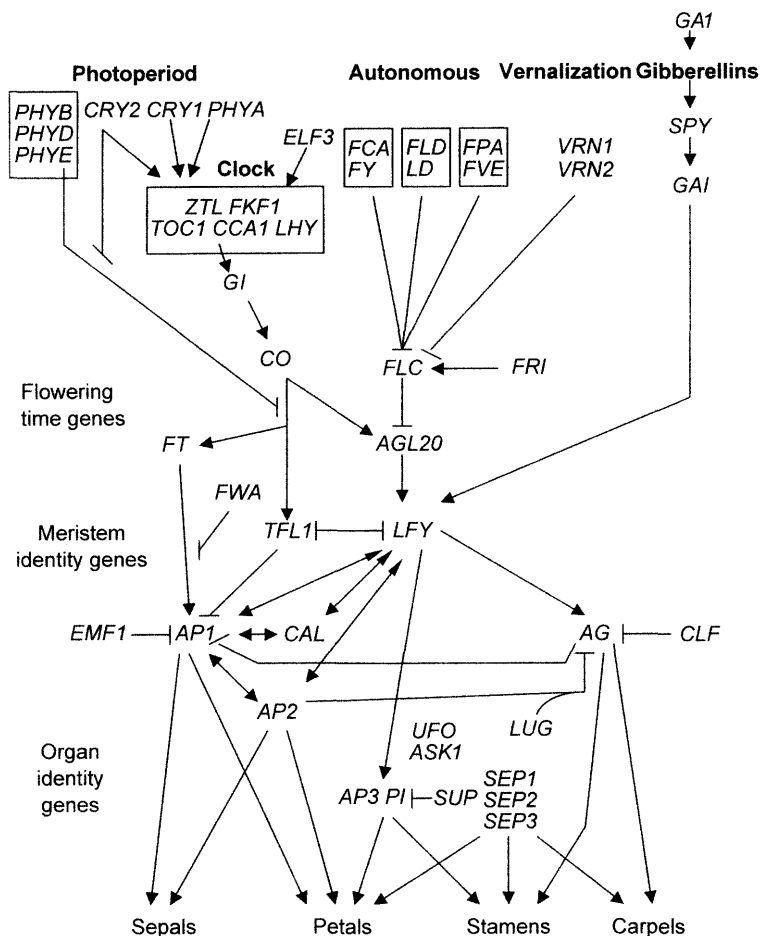


Figure 4.4 An illustration of genetic interactions among genes regulating flowering time and floral structures. Positive interactions are indicated by arrows and negatives ones by a line with a short bar at the end. (This figure is modified from those in Theissen and Saedler, 1999; and Blazquez, 2000.)

Whitelam *et al.*, 1993) cause long hypocotyls in far-red light and a reduction of flowering promotion due to day length extension or night interruption (Bagnall *et al.*, 1995). Furthermore, overexpression of the *Arabidopsis* *PHYA* gene promotes early flowering. In contrast, *PHYB* is relatively light stable and the predominant red receptor; *phyB* mutants flower early, indicating that *PHYB* mediates the inhibition of flowering by red light (Devlin *et al.*, 1996). *phyB* mutants can still respond to long days, probably because of the presence of other light-stable phytochromes, *PHYC* to *PHYE*. Support for this hypothesis has come from the recent observation that mutations in the *PHYD* gene, which

encodes a protein with approximately 80% amino acid sequence identity to PHYB, enhance the early-flowering phenotype of *phyB* mutants (Aukerman *et al.*, 1997; Devlin *et al.*, 1999). Similarly, a mutation in *PHYE* was found to cause early flowering in the *phyA phyB* double mutant (Devlin *et al.*, 1998).

Plants also respond to blue light, which is important for the regulation of the circadian clock. In *Arabidopsis*, there are two genes, *CRY1* and *CRY2*, which encode photolyase-like blue light receptors called cryptochromes (Lin, 2000). *CRY1* (previously called *HY4*; Ahmad and Cashmore, 1993) is important for the inhibition of hypocotyl elongation under blue light and is involved in regulating the circadian clock. In addition, *PHYA* may also mediate blue light responses, since both *cry1* and *phyA* mutants display extended period length under blue light in the oscillation of circadian-clock-regulated gene expression (Lin, 2000). The *cry2* mutants have delayed flowering under long days, and molecular genetic analyses indicate that the *CYR2* gene is the same as the late-flowering gene, *FHA* (Guo *et al.*, 1998). Furthermore, the overexpression of *CRY2* caused slightly accelerated flowering under short days but not long days, supporting the idea that *CYR2* is important for the long-day pathway. Further genetic analysis suggests that *CRY2* promotes flowering by antagonizing the negative effect of PHYB (Guo *et al.*, 1998; Mockler *et al.*, 1999). In addition, *CRY1* and *CRY2* act redundantly to promote flowering in a PHYB-independent manner (Mockler *et al.*, 1999).

Both phytochromes and cryptochromes appear to function in the nucleus, with the former being transported into the nucleus in a light-dependent fashion (Guo *et al.*, 1999; Kircher *et al.*, 1999). Recently, the PHYB and CRY2 proteins were found to interact by co-immunoprecipitation. Fluorescent resonance energy transfer with fusion proteins in living cells suggest that the interaction between PHYB and CRY2 is light-dependent (Mas *et al.*, 2000). One model suggested by these results is that CRY2 antagonizes PHYB activity via a direct physical interaction.

In addition to the photoreceptors, other genes have been identified that play critical roles in photocontrol of the circadian clock. Mutations in the *ZTL* gene were found to lengthen the free-running period of clock-controlled gene transcription and to alter the day-length-dependent flowering time (Somers *et al.*, 2000). In addition, overexpression of *ZTL* (also called *LKP1*; Kiyosue and Wada, 2000) causes late flowering under long days but not short days (Kiyosue and Wada, 2000). Mutations in another gene, *FKF1*, also caused defects in clock-controlled gene expression and delayed flowering (Nelson *et al.*, 2000). Both *ZTL* and *FKF1* genes encode similar proteins with a PAS/LOV domain, an F-box and six kelch repeats. PAS/LOV domains are found in other proteins mediating light responses, such as the phototropin (NPH1) protein of *Arabidopsis* that is required for blue-light regulated phototropism (Liscum and Briggs, 1995; Huala *et al.*, 1997). The LOV domain in NPH1 has been shown to bind to flavin and is thought to respond to light directly (Christie *et al.*, 1998, 1999). The F-box is a

motif that mediates ubiquitination of proteolytic targets (Skowrya *et al.*, 1997), and kelch repeats may be involved in protein-protein interactions (Adams *et al.*, 2000). Therefore, *ZTL* and *FKF1* may control the circadian clock by mediating light regulated protein-protein interactions and/or protein degradation.

Molecular genetic analyses have also revealed additional genes that regulate the circadian clock. Mutants defective in the *ELF3* gene are early-flowering in a photoperiod insensitive way, and are arrhythmic under constant light conditions but not constant dark or light/dark conditions (Hicks *et al.*, 1996; Zagotta *et al.*, 1996). These observations suggest that the *elf3* mutant is defective in the input of light signal to the circadian clock, but not in the clock pacemaker in the dark. Further genetic studies suggest that *ELF3* is independent of *PHYB* and probably functions in a separate pathway (Reed *et al.*, 2000), with the *ELF3* protein regulating light signaling to the circadian clock (McWatters *et al.*, 2000). Another mutation, *toc1*, was found to shorten the circadian rhythms in light-grown plants and affect photoperiodic control of flowering (Somers *et al.*, 1998). The *TOC1* gene encodes a protein with domains similar to the receiver domain of the two-component system response-regulator and to motifs involved in transcriptional regulation (Strayer *et al.*, 2000). *TOC1* is expressed in a clock-regulated fashion and is part of a feedback loop suggesting that *TOC1* may mediate signal input of the clock machinery.

Other genes may be more intimately associated with the clock. A dominant mutation in the *LHY* gene disrupts the circadian clock regulation of gene expression and causes photoperiod-independent flowering (Schaffer *et al.*, 1998). In addition, the *CCA1* gene is involved in the phytochrome induction of gene expression (Wang and Tobin, 1998). Expression of both *LHY* and *CCA1* is regulated by the clock, and constitutive expression of either one of them abolishes the circadian rhythm of clock-regulated genes, including that of the endogenous *LHY* and *CCA1* genes. Furthermore, both *LHY* and *CCA1* genes encode MYB-domain containing proteins, suggesting that they are transcriptional regulators. These properties support the hypothesis that these two genes are involved in a feedback loop that perhaps maintains the circadian clock, and that at least part of this loop requires transcriptional control.

While the similarity of both *LHY* and *CCA1* to Myb-domain factors suggests that transcriptional control is important in the regulation of the photoperiodic pathway, other mechanisms of regulation are clearly important, as indicated by the results from experiments with the CK2 protein kinase. CK2 interacts with the *CCA1* and *LHY* proteins *in vitro* and phosphorylates *LHY*. Furthermore, overexpression of *CK2* causes shortened periods of rhythmic expression of *CCA1* and *LHY* and early flowering under both long and short days, suggesting a defect in the photoperiod/clock control of flowering time (Sugano *et al.*, 1999). Another gene that seems to participate in this regulatory loop is *GI*, which is required for the long-day acceleration of flowering and encodes a putative transmembrane protein (Fowler *et al.*, 1999; Park *et al.*, 1999). *GI* expression

is altered in the recessive *elf3* and dominant *lhy* mutants, as well as plants overexpressing *CCA1*; at the same time, *LHY* and *CCA1* expression is reduced in *gi* mutants (Fowler *et al.*, 1999; Park *et al.*, 1999). Furthermore, although GI seems to be a nuclear protein that mediates phytochrome signaling (Huq *et al.*, 2000), its similarity to transmembrane proteins suggests that it is unlikely to be a transcription factor. Because phytochromes and cryptochromes both function in the nucleus, it is possible that GI serves to regulate protein localization to the nuclear membrane.

A central effector of long-day signaling/circadian clock is the *CO* gene, which encodes a zinc-finger transcription factor (Putterill *et al.*, 1995). *CO* is expressed at a higher level under long days than short days; in addition, the expression of *CO* is positively regulated by the blue-light receptor, CRY2 (Guo *et al.*, 1998). Furthermore, overexpression of *CO* using the 35S promoter causes early flowering regardless of the actual day length (Pineiro and Coupland, 1998). Therefore, *CO* appears to act late in the long-day promotion of flowering. In addition, it appears to be one of the only known genes whose function is limited to this pathway, and thus can be contrasted with photoreceptors and circadian clock components that mediate several processes.

Homologs of *CO* have now been isolated from several plants (Moon *et al.*, 1998; Robert *et al.*, 1998; Lagercrantz and Axelsson, 2000). In addition, a *Brassica* *CO* homolog was able to rescue the *co* mutant of *Arabidopsis*, suggesting that the *CO* function has been conserved between *Arabidopsis* and *Brassica* (Robert *et al.*, 1998). Moreover, a major quantitative trait locus (QTL) in rice controlling photoperiod sensitivity, *Hd1*, has recently been demonstrated to be homologous to the *CO* gene (Yano *et al.*, 2000). However, the activity of *Hd1* is intriguing in that, in contrast to *Arabidopsis*, rice is a short-day flowering plant, its heading normally promoted by short days. The *Hd1* gene from a photoperiod responsive strain was able to confer photoperiod sensitivity when introduced into a non-responsive strain by transformation. Furthermore, *sel* mutations that reduce photoperiod sensitivity were found to be alleles of *Hd1* (Yano *et al.*, 2000). Expression analysis indicates that the expression of *Hd1* is not dramatically affected by photoperiod, unlike that of *CO*. It will be interesting to learn whether under short days *Hd1* activates expression of floral meristem genes, such as rice homologs of *LFY* and *API*.

4.3.3 The autonomous and vernalization pathways

Another group of genes from *Arabidopsis*, *FCA*, *FY*, *FPA*, *FVE*, *LD* and *FLD*, promote flowering under both long and short days. These genes were initially identified by mutations that cause late flowering independent of day length (Koornneef *et al.*, 1991, 1998; Simpson *et al.*, 1999). Double mutant analyses suggest that these genes are not in a linear pathway; *FCA* and *FY* seem to be in one pathway, and *FPA* and *FVE* in another. However, the lack of information

on whether many of the mutations are null means that the relationship between these genes remains uncertain. Another variation is that the *ld* and *fld* mutations are only effective in ecotypes with a strong *FLC* allele (see section 4.3.5). Molecular cloning has revealed that the *LD* gene encodes a putative transcription factor containing a homeodomain (Lee *et al.*, 1994). In addition, *FCA* encodes a putative RNA-binding protein (Macknight *et al.*, 1997) with a WW domain thought to mediate protein-protein interaction. Overexpression of *FCA* leads to early flowering; therefore, the amount of *FCA* transcripts is normally limiting.

Genetic studies in maize have identified the *indeterminate1* (*id1*) mutation that delays flowering (Colasanti *et al.*, 1998; McSteen *et al.*, 2000). The *id1* mutant produces many more leaves than the wild-type plant, and even after the mutants produce reproductive shoots, they still have vegetative properties. It is thought that *ID1* may act in an autonomous pathway, although this is not conclusive (McSteen *et al.*, 2000). The *ID1* gene product is similar to zinc-finger transcription factors (Colasanti *et al.*, 1998), suggesting that it controls other genes. Furthermore, *ID1* is expressed in young leaves, but not at the shoot apex, suggesting that it may regulate the level of a transmittable substance that indirectly controls flowering transition (Colasanti *et al.*, 1998; Ma, 1998a). Although both CO and ID1 are zinc-finger proteins, they are not homologs. Putative zinc-finger proteins that are very similar to ID1 have been revealed by the *Arabidopsis* genome sequencing project, but their functions are not known.

In many plants that naturally grow at high latitudes, a prolonged treatment of cold temperature, or vernalization, followed by warmer temperature will accelerate flowering. This is also true for *Arabidopsis* ecotypes that are found in regions with cold winters, such as northern Europe (Simpson *et al.*, 1999). Vernalization can reverse the effects of mutations in the autonomous pathways, such as *fca* and *fpa*, and the vernalization mutants, *vrn1* and *vrn2*, were isolated by reduced response in the *fca* mutant background (Chandler *et al.*, 1996). Another mutation, *efs*, was isolated as an early flowering mutant in the *fwa* mutant background (Soppe *et al.*, 1999). Further genetic analysis has indicated that it also suppresses the late flowering phenotypes of *fca* and *fve* mutations in the autonomous pathway. The *efs* mutant also failed to respond to vernalization, suggesting that it is involved in this pathway.

It has been proposed that DNA demethylation is a consequence of vernalization, which leads to gene activation that in turn promotes flowering (Burn *et al.*, 1993). This was tested using DNA methyltransferase gene (*MET1*) antisense plants, which showed early flowering but still had some response to vernalization (Finnegan *et al.*, 1996, 1998; Ronemus *et al.*, 1996). Hypomethylation at specific loci could also cause late flowering (Kakutani, 1997). The residual vernalization response could be due to incomplete demethylation in the antisense plants.

4.3.4 The gibberellic acid (GA) and abscisic acid (ABA) pathways

GA promotes flowering and is required for flowering in *Arabidopsis* under short days, when the long-day pathway is inactive (Simpson *et al.*, 1999). Under short days, the *gal* mutant defective in GA biosynthesis cannot flower without exogenous GA, and the dominant *gai* mutant that is defective in GA response flowers extremely late. Further genetic analyses of *gal* mutant in combination with other mutations and through vernalization treatments have supported the notion that the GA pathway is separate from the autonomous and vernalization pathways (Michaels and Amasino, 1999a).

Cloning of the *GAI* has revealed that it encodes a putative transcription factor that is thought to negatively regulate the GA response (Peng *et al.*, 1997). The *RGAI* gene, identified as a recessive mutation that suppressed the *gal* phenotype, also encodes a related putative transcriptional regulator (Silverstone *et al.*, 1997, 1998). Recessive mutations in either *GAI* or *RGAI* do not cause a defect in GA response or flowering, indicating that these genes are functionally redundant. Furthermore, the *spy* mutant exhibits constitutive GA response and flowers early (Jacobsen and Olszewski, 1993; Jacobsen *et al.*, 1996), suggesting that *SPY* is also a negative regulator of the GA response. *spy* mutations are epistatic to the *gai* mutation, suggesting that *SPY* act downstream of *GAI*. The *SPY* gene encodes protein with a tetratricopeptide repeat region, which can mediate protein-protein interactions (Blatch and Lassel, 1999). Exogenous GA can induce flowering in the *vrn1* mutant, indicating that GA and vernalization control separate pathways (Chandler *et al.*, 1996).

Another gene, *FPP1*, was identified as a gene that is expressed in the apical meristem immediately following photoperiod induction (Kania *et al.*, 1997). Constitutive expression of *FPP1* causes early flowering in several late-flowering mutants in both autonomous and long-day pathways, including *fca*, *fve*, *gi*, *co* and *fwa* (Melzer *et al.*, 1999). Interestingly, analyses using GA deficient mutant and a GA biosynthesis inhibitor suggest that *FPP1* is involved in mediating GA signaling. If true, this would provide a link between the long-day pathway and the GA pathway. In addition, a day-length-independent early-flowering mutant, *eafl*, was found to have increased resistance to a GA biosynthesis inhibitor, suggesting that it is also involved in GA response (Scott *et al.*, 1999).

The hormone, abscisic acid, is known to repress flowering under short days (Martinez-Zapater *et al.*, 1994), and mutants defective in ABA synthesis or response flower early under short days. Recent observations that vernalization was normal in *abi1*, *abi2* and *abi3* mutants suggest that ABA does not control the vernalization pathway (Chandler *et al.*, 2000). Furthermore, double mutants of *abi1* or *abi2* with *fca* showed earlier flowering than the *fca* single mutant, indicating that the ABA-stimulated repression is still operating when the autonomous pathway is defective. ABA is known to antagonize GA in other

responses, such as seed germination (Finkelstein and Zeevaart, 1994), but the relationship between GA and ABA pathways in regulating flowering time is not known, particularly under short days.

4.3.5 Integration of flowering pathways

Genes downstream of two or more pathways have been identified by mutant analysis and ecotype comparisons (Simpson *et al.*, 1999). The *FT* and *FWA* genes were originally identified as late-flowering mutants, and genetic studies placed them in the long-day pathway because they do not have dramatic effect under short days (Koornneef *et al.*, 1991; Martinez-Zapater *et al.*, 1994). More recently, it was proposed that *FT* and *FWA* functioned differently from genes in the long-day pathway, such as *CO*, because *fwa* and *ft* mutants do exhibit minor delays under short days (Simpson *et al.*, 1999). Nevertheless, a screen for mutations that suppress the early-flowering phenotypes due to constitutive *CO* expression has recovered new alleles of the *FT* and *FWA* genes (Onouchi *et al.*, 2000), suggesting that *FT* and *FWA* could either be downstream of or in parallel with *CO*.

The *FT* gene has been isolated both by activation tagging and by T-DNA tagging (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). *FT* is expressed in all tissues of the seedling and mature plants, with its level increasing gradually under both long and short days. The upregulation of *FT* expression is delayed in *co* mutants, but not affected in *fha* (*cyr2*), *fca* or *fwa* mutants. Constitutive *FT* expression causes *CO*-independent early flowering under both long and short days, supporting the idea that *FT* is downstream of *CO*. In contrast, the semi-dominant *fwa-2* mutation partially suppressed the early flowering effects of constitutive *FT* expression, suggesting that *FWA* may act at the same level as, or downstream of, *FT*. Therefore, *CO*, *FWA* and *FT* do not necessarily form a linear pathway. *FT* encodes a protein (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) similar to phosphatidylethanolamine-binding proteins, which are thought to be involved in signal transduction (Banfield *et al.*, 1998). Recently, a phosphatidylethanolamine-binding protein (RKIP) was found to interact with and inhibit the Raf kinase that activates the MAP kinase signaling pathway (Yeung *et al.*, 1999), providing direct evidence for a role in signaling for this family of proteins.

The *FWA* gene has been further studied using molecular genetic techniques (Soppe *et al.*, 2000). The original *fwa* mutants are semi-dominant, suggesting that they might be gain-of-function alleles. Alternatively, the semi-dominance might be a reflection of haplo-insufficiency. To distinguish these possibilities, intragenic suppressors of *fwa-1* were screened for lines that had a less severe (wild-type-like) phenotype (Soppe *et al.*, 2000). Three suppressors with normal flowering time were obtained and mapped to the *FWA* locus, supporting the hypothesis that the original mutation was a gain-of-function allele.

The *FWA* gene encodes a homeodomain protein (Soppe *et al.*, 2000). Northern hybridizations indicate that, in the original semi-dominant mutants, it is expressed at different stages and in several organs including flowers, but it is not detectable in the wild-type or the revertants. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that the wild-type *FWA* gene is expressed in siliques, but not in seedlings, vegetative plants or flowers. The DNA sequences of the *fwa-1* and *fwa-2* alleles are identical to the wild-type sequence, suggesting the *fwa-1* and *fwa-2* alleles are due to an epigenetic change that caused an elevated level of expression. It was observed that *FWA* expression is not elevated in *fca*, *fve*, *co*, *gi* or *ft* mutants. Therefore, *FWA* is probably not downstream of these genes. In light of these data and the realization that *fwa-1* is a gain-of-function mutation, the suppression of the early flowering phenotypes of 35S-*CO* and (partially) of 35S-*FT* can be reinterpreted as a counter effect by the *FWA* function in promoting vegetative development. However, the fact that the revertants carry loss-of-function mutations and have normal flowering time indicates that *FWA* is not required for regulating this transition.

Flowering time genes have also been identified by comparing allelic difference between different ecotypes having distinct flowering behaviors (e.g. Lee *et al.*, 1993; Clarke and Dean, 1994; Alonso-Blanco *et al.*, 1998; Swarup *et al.*, 1999), leading to the identification of *FLC* and *FRI* (Simpson *et al.*, 1999). For both genes, late-flowering alleles are dominant over early-flowering ones, suggesting that the gene functions to repress flowering. The hypothesis that the dominant late-flowering allele of the *FLC* gene is functional is supported by the isolation of *flc* null alleles that confer early flowering phenotypes (Michaels and Amasino, 1999b). The *FLC* gene has been isolated and it encodes a MADS-domain protein (Michaels and Amasino, 1999b). Genetic interactions and the observation that the levels of *FLC* mRNA increase in mutants of the autonomous pathway, such as *fca*, *fpa* and *ld*, suggest that the autonomous pathway represses *FLC* expression (Sanda and Amasino, 1996; Michael and Amasino, 1999b). Vernalization also represses *FLC* expression in an *FRI*-independent way, suggesting that *FLC* integrates both the autonomous and vernalization pathways (Michaels and Amasino, 1999b; Sheldon *et al.*, 1999, 2000).

When dominant *FRI* and *FLC* alleles are combined, plants flower much later than when either gene is functioning separately. Genetic and molecular studies support the idea that *FLC* represses flowering and *FRI* enhances *FLC* expression (Michaels and Amasino, 1999b). Map-based cloning of the *FRI* reveals a novel protein with predicted coiled coil regions (Johanson *et al.*, 2000). Sequence analysis indicates that the recessive alleles of *FRI* in *Ler* and *Col* ecotypes carry mutations that either cause a frameshift or delete the promoter region, making them likely null alleles.

A recently characterized gene, *SVP*, was identified as an early-flowering mutant in *Arabidopsis* (Hartmann *et al.*, 2000). Cloning of the gene by transposon tagging reveals a MADS-domain protein. The *SVP* gene is expressed as

several different sized transcripts in vegetative tissues and floral primordia, but not in mature flowers or siliques. The expression pattern is consistent with its role as a repressor of flowering, and the presence of several transcripts suggest possible alternative splicing, which was also observed for *FCA* (Macknight *et al.*, 1997).

Another MADS-box gene, *AGL20*, has been identified as a gene promoting flowering by several independent lines of evidence (Borner *et al.*, 2000; Lee *et al.*, 2000; Onouchi *et al.*, 2000; Samach *et al.*, 2000). By use of activation tagging, it was found that overexpression of *AGL20* can promote early flowering (Lee *et al.*, 2000). In contrast, transposon insertions in the *AGL20* gene were found to cause late flowering (Borner *et al.*, 2000). Further analysis has indicated that *AGL20* mRNA is reduced in late-flowering backgrounds with the dominant *FLC* allele, indicating that *AGL20* is downstream of *FLC*. In addition, a mutation in *AGL20*, *soc1*, was isolated as a suppressor of the early flowering phenotype of the constitutive *CO* plants (Onouchi *et al.*, 2000; Samach *et al.*, 2000). This might suggest that *AGL20* is downstream of *CO* and the long-day pathway. However, *AGL20* expression is only slightly reduced in the *co* mutant, leaving open the possibility that the suppression of 35S-*CO* by the *soc1* mutation is an indirect effect. In *Arabidopsis*, it is not clear what the relationship between *FWA*, *FT* and *AGL20* is, nor how the GA pathway can be integrated with the other pathways (see section 5). A putative homologue of *AGL20* (*saMADSA*) has been isolated from *Sinapis alba*, and its expression is activated by cytokinins and GA, suggesting that it might mediate regulation of flowering via these signal (Bonhomme *et al.*, 2000).

The foregoing discussion emphasizes how the response of plants to both developmental and environmental cues to achieve an optimal time for flowering necessitates a complex regulatory network. The elements of this network and their interactions are only beginning to be uncovered. The present discussion represents a rather simplistic view and is aimed at providing a brief background for subsequent debate concerning genes controlling meristem identity and their regulation by the flowering time genes.

4.4 Specification of floral meristem identity

When cells form a new meristem, they can either remain as the previous meristem type or become a new type. Specifically, cells that form the floral meristem must decide to switch cell fate to become the more specialized and determinate meristem. Genetic studies in *Antirrhinum* and *Arabidopsis* have uncovered two groups of genes with opposing functions (Ma, 1998b) (table 4.2 and figure 4.4): one set of genes, called floral meristem identity genes, promote floral meristem identity, whereas another gene acts to maintain the inflorescence meristem identity. In this section, the function of these two kinds of genes is briefly described

Table 4.2 *Arabidopsis* genes affecting reproductive meristem formation and function

Gene	Phenotype ^a	Protein function
<i>AG</i>	defect in stamen and carpel; more sets of sepals and petals	MADS-box; transcription factor
<i>ANT</i>	defect in integument development; reduced FON	AP2-repeat; transcription factor
<i>API</i>	inflorescence transformation defect; defect in sepal and petal, reduced FON	MADS-box; transcription factor
<i>AP2</i>	abnormal sepal and petal; reduced FON	AP2-repeat; transcription factor
<i>AP3</i>	defect in petal and stamen; slightly reduced FON	MADS-box; transcription factor
<i>ASK1</i>	abnormal petal, slight reduction of petal and stamen number	SCF ubiquitin ligase subunit
<i>CAF</i>	extra number of stamens and variable number of carpels	RNA helicase and RNase III
<i>CAL</i>	enhancing <i>apl</i> phenotypes	MADS-box; transcription factor
<i>CLF</i>	early flowering; floral organ defects	SET Polycomb group protein
<i>CLV1</i>	extra large SAM and increased FON	receptor-linked kinase
<i>CLV2</i>	extra large SAM and increased FON	receptor-like protein
<i>CLV3</i>	extra large SAM and increased FON	peptide ligand
<i>ETT</i>	more sepal and petal; fewer stamens and abnormal gynoecium	transcription factor
<i>FFO1,2,3</i>	fusion of floral organs; enhancing <i>ufo</i> phenotypes	
<i>FIL</i>	filamentous structure and changed floral organ number and shape	transcription factor
<i>FUL</i>	meristem identity (redundant with <i>API</i> and <i>CAL</i>); fruit development	MADS-box; transcription factor
<i>LFY</i>	inflorescence transformation defect; decreased FON	transcription factor
<i>LUG</i>	abnormal sepal and petal; reduction of FON	WD40-repeat; transcriptional co-repressor factor
<i>MGO1</i>	large SAM: fasciation of inflorescence stem and reduced FON	
<i>MGO2</i>	large SAM: fasciation of inflorescence stem and reduced FON	
<i>PAN</i>	five sepals, petals and stamens	b-ZIP; transcription factor
<i>PI</i>	defect in petal and stamen; slightly reduced FON	MADS-box transcription factor
<i>PTL</i>	defect in petal initiation; reduced petal number	
<i>SEP1/2/3</i>	flower with only sepals	MADS-box transcription factors
<i>STM</i>	absent or reduced SAM; reduced FON	homeodomain protein
<i>SUP</i>	extra number of stamens; reduced number of carpels	zinc finger protein
<i>TFL1</i>	early flowering: terminal flower	phosphatidylethanolamine- binding protein
<i>TFL2</i>	terminal flower	

Table 4.2 (continued)

Gene	Phenotype ^a	Protein function
<i>TSL</i>	reduced FON; abnormal gynoeceum	protein kinase
<i>TSO1</i>	increased number of sepals; without petals, stamens, carpels	cysteine-rich repeats protein
<i>UFO</i>	defect in floral organ and meristem; slightly reduced FON	F-box protein, SCF subunit
<i>WIG</i>	large SAM and increased FON	farnesyltransferase subunit
<i>WUS</i>	reduced SAM and reduced FON	homeodomain protein
<i>ZLL</i>	abnormal SAM	novel protein

^aPhenotype of recessive mutant, unless noted otherwise. Abbreviations: FON, flower organ number; SAM, shoot apical meristem.

and their interactions are discussed. In addition, several genes involved in the control of the determinacy of floral meristems are discussed as well as additional genes that contribute to floral meristem identity and development.

4.4.1 *The floral meristem identity genes, LFY/FLO, AP1/SQUA and CAL*

Several genes have been identified in *Arabidopsis* and *Antirrhinum* that control the floral meristem identity (Ma, 1998b). These floral meristem identity genes include: *LEAFY* (*LFY*), *APETALA1* (*API*) and *CAULIFLOWER* (*CAL*) in *Arabidopsis* (Mandel *et al.*, 1992; Weigel *et al.*, 1992; Kempin *et al.*, 1995), and *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*) in *Antirrhinum* (Coen *et al.*, 1990; Huijser *et al.*, 1992). Inactivation of a gene in this group generally causes the replacement of individual flowers with shoots, or with structures that combine features of flowers and shoots.

In *Arabidopsis*, *LFY* plays a critical role in the specification of floral meristem identity. Loss-of-function *lfy* mutants produce shoots in place of early-arising flowers, and late-arising flowers have features of the inflorescence, indicating a partial defect in the transition from inflorescence to floral meristem (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel *et al.*, 1992). For example, most of the *lfy* floral organs are leaf-like or sepal-like and arranged in a spiral rather than in whorls, similar to vegetative shoots. In addition, axillary flowers sometimes develop within the *lfy* mutant flowers. These phenotypes are similar to the *flo* mutants of *Antirrhinum*, which produce vegetative shoots instead of flowers, exhibiting a complete failure to specify floral meristem (Coen *et al.*, 1990).

Both *FLO* and *LFY* genes have been isolated and shown to encode homologous transcription factors (Coen *et al.*, 1990; Weigel *et al.*, 1992), which are conserved in and unique to plants (Frohlich and Eastabrook, 2000; Theissen,

2000). In wild-type plants, *LFY* expression precedes that of other meristem identity genes with flower-specific expression (Weigel *et al.*, 1992). Although the highest expression of *LFY* is found in regions of the inflorescence meristem that will form floral meristems, and in newly formed floral meristems, *LFY* is also expressed at low levels during vegetative development (Weigel *et al.*, 1992; Blazquez *et al.*, 1997). *LFY* expression in the wild-type is rapidly elevated under long days and gradually increased under short days (Blazquez *et al.*, 1997). Overexpression of *LFY* causes precocious flowering and the conversion of lateral inflorescence shoots into flowers in transgenic plant (Weigel and Nilsson, 1995). Therefore, the level of *LFY* expression is directly correlated with its ability to induce flower development.

FLO/LFY homologues have also been isolated from several eudicotyledonous plants (Anthony *et al.*, 1993; Kelly *et al.*, 1995; Hofer *et al.*, 1997; Pouteau *et al.*, 1997; Kyozyuka *et al.*, 1998; Souer *et al.*, 1998; Southerton *et al.*, 1998; Rottmann *et al.*, 2000; Shu *et al.*, 2000). In particular, the tomato *falsiflora* mutant is defective in the *FLO/LFY* homolog (Molinero-Rosales *et al.*, 1999). Like *flo* and *lfy* mutants, the *falsiflora* mutant also produces inflorescence shoots in place of flowers, indicating a defect in specification of floral meristem identity. Furthermore, the mutant is late flowering and produces more leaves. In pea, the *unifoliata* (*uni*) mutant lacking the *FLO/LFY* homologous gene function is abnormal in floral identity, like the *flo* and *lfy* mutants. In addition, the *uni* mutant produces a single leaflet instead of the normal compound leaf, suggesting a broader range of function for the *UNI* gene (Hofer *et al.*, 1997; see also chapter 7 of this volume).

When introduced into rice by transformation, *LFY* causes early heading (He *et al.*, 2000), suggesting that a *LFY*-like activity normally promotes flowering in rice. In addition, a putative *FLO/LFY* homolog (*RFL*) has been isolated from rice, which is highly expressed in young leaves and panicles (a branch type of the inflorescence) including the apical meristem, but not in the incipient primary branches or mature florets (Kyozyuka *et al.*, 1998). Constitutive expression of *RFL* in *Arabidopsis* causes pleiotropic developmental defects in leaves and floral organs, but not the conversion of the inflorescence meristem to a floral meristem seen in plants constitutively expressing *LFY*. This suggests that *RFL* might have a different function from that of *FLO*, *LFY* and their homologs in eudicotyledonous plants, although further studies using mutants or transgenic plants in rice are needed. Interestingly, several gymnosperm species, including pine (*Pinus radiata*) and Gingko, have two distant homologs of *LFY* (Mellerowicz *et al.*, 1998; Mouradov *et al.*, 1999; Frohlich and Eastbrook, 2000). Furthermore, the *NEEDLY* gene from the radiata pine can function like *LFY* when introduced into *Arabidopsis* (Mouradov *et al.*, 1999).

The *Arabidopsis* *API* gene is also an important gene promoting floral meristem identity (Irish and Sussex, 1990; Mandel *et al.*, 1992; Bowman *et al.*, 1993). The loss-of-function mutants of *API* produce shoots in place of early-arising

flowers and exhibit defects in floral meristems of late-arising flowers, indicating that the transition from inflorescence to floral meristems is not normal. Although *ap1* mutants produce flowers with axillary flowers, these flowers produce fully functional reproductive organs, indicating that they retain considerable floral characteristics. In *Antirrhinum*, the transition from inflorescence to flower development involves the action of the *API* homolog *SQUAMOSA* (*SQUA*) gene, where flowers in *squa* mutants are replaced by shoots that bear occasional flowers (Huijser *et al.*, 1992). Therefore, *ap1* and *squa* mutants exhibit weaker phenotypes than *lfy* and *flo* mutants, respectively.

The *API* gene encodes a MADS-domain transcription factor and is expressed throughout the newly formed floral meristem (Mandel *et al.*, 1992). Therefore, *API* expression occurs after the *LFY* gene is already highly expressed. Similarly, the *SQUA* gene encodes a MADS-domain protein homologous to *API*, and is expressed in early floral meristems (Huijser *et al.*, 1992). In addition, constitutive expression using a 35S-*API* construct in transgenic plants caused early flowering, the premature termination of the primary inflorescence, and the conversion of lateral inflorescences to single flowers (Mandel and Yanofsky, 1995a). Putative *API/SQUA* homologs have been isolated from several plants, and their expression patterns are consistent with the idea that they play regulatory roles in early flower development (Mena *et al.*, 1995; Kyoizuka *et al.*, 1997; Lowman and Purugganan, 1999). However, because *API* is in a large clade of the MADS-box gene family with many very similar genes (Purugganan *et al.*, 1995; Purugganan, 1997), determining whether these genes are true homologs of *API* will require further functional studies using molecular genetic techniques.

In addition to *LFY* and *API*, the *CAULIFLOWER* (*CAL*) gene also contributes to floral meristem identity, although the *cal* single mutant has no effect on meristem identity (Bowman *et al.*, 1993; Kempin *et al.*, 1995). Nevertheless, *cal-1* enhances the effects of *ap1* mutation in *cal-1 ap1* double mutants, indicating that *CAL* is functionally redundant with *API* (Bowman *et al.*, 1993). *CAL* encodes a MADS-domain transcription factor that is very similar to *API* (76% in amino acid sequence identity; Kempin *et al.*, 1995). In addition, the expression pattern of *CAL* is similar to that of *API* in young floral primordia.

The *FUL* gene (also called *AGL8*), a MADS-box gene expressed in the inflorescence meristem, is negatively regulated by *API* (Mandel and Yanofsky, 1995b). A *ful* single mutant has no meristem defects; however, when the *ful* mutation was combined with *ap1* and *cal* mutations, it was discovered that *FUL* has a redundant function in regulating meristem identity (Gu *et al.*, 1998; Ferrandiz *et al.*, 2000). It seems that ectopic *FUL* expression in the reproductive meristem of the *ap1 cal* double mutant can play a role in promoting meristem activity, even though *FUL* is not normally expressed in the floral meristem.

In maize, several mutants have been isolated that alter the identities of reproductive meristems (McSteen *et al.*, 2000). For instance, in the *ramosa1* and

ramosa2 mutants, the spikelet pair (short branch) or spikelet is replaced with a long branch. In addition, the *branched silkless1* mutant reiterates spikelet-like meristem instead of producing a floret meristem. These mutants might be defective in genes that are homologous to *LFY* or *API*. Furthermore, two maize MADS-box genes, *ZMM8* and *ZMM14*, exhibit differential expression between the two florets within the maize spikelet, suggesting that they may play a role in specifying the functional difference of the florets (Cacharrn *et al.*, 1999).

4.4.2 Genes promoting the inflorescence identity

In contrast to *LFY/FLO* and *API/SQUA* genes, the *TERMINAL FLOWER1* (*TFL1*) in *Arabidopsis* (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992; Bradley *et al.*, 1997) and *CENTRORADIALIS* (*CEN*) in *Antirrhinum* (Carpenter and Coen, 1990; Bradley *et al.*, 1996) act to maintain the inflorescence meristem. In *tfl1* mutant plants, the primary inflorescence terminates with a terminal flower after producing a small number of flowers (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992). In addition, the lateral inflorescences are replaced by a single flower. Therefore, the inflorescence meristems in *tfl1* mutants are converted to floral meristems. Similarly, *cen* mutants produce a terminal flower at the apex of the inflorescence after the production of only several lateral flowers (Carpenter and Coen, 1990; Bradley *et al.*, 1996). The fact that lateral flowers are produced initially in the *cen* and *tfl1* primary inflorescences indicates that the inflorescence meristem is active during early reproductive development.

Isolation of the *CEN* gene by transposon tagging reveals an encoded protein similar to phosphatidylethanolamine binding proteins (Bradley *et al.*, 1996). The *TFL1* was cloned using the *CEN* as a probe and by positional cloning, and its protein product is about 70% similar to *CEN* (Bradley *et al.*, 1997; Ohshima *et al.*, 1997). In wild-type plants, both *CEN* and *TFL1* are expressed in a central region of the inflorescence meristem, separate from the regions of *FLO/LFY* and *SQUA/API* expression (Bradley *et al.*, 1997; Ratcliffe *et al.*, 1999). The similarity of *CEN* and *TFL1* to *RKIP* (Yeung *et al.*, 1999) suggests that they might play a role in signaling via the MAP kinase cascade.

Because *CEN* and *TFL1* affect inflorescence architecture in *Antirrhinum* and *Arabidopsis*, it is instructive to investigate their homologs in other plants with different inflorescence structures. In particular, when the *CEN* genes were introduced into tobacco, which normally develops a determinate inflorescence with a terminal flower, the vegetative phase was extended and flowering was delayed (Amaya *et al.*, 1999). Furthermore, endogenous *CEN*-like genes (*CET*) are highly expressed in the vegetative axillary meristem but not in the main shoot meristem. These results suggest that the *CET* genes also regulate shoot structure, although their precise roles are different from those of *CEN* and *TFL1*. In the tomato *self-pruning* (*sp*) mutant, a defective *CEN/TFL1* homolog leads to termination of shoot development with two consecutive inflorescences.

unlike the normal shoots with inflorescences separated by three vegetative nodes (Pnueli *et al.*, 1998). Furthermore, *SP* expression from the 35S promoter can restore the vegetative-reproductive alternation in the *sp* mutant and increase the number of vegetative nodes in one of the tomato lines with normal *SP* function. These observations suggest that the genes controlling the complex alternation between vegetative and reproductive development in tomato may be related to those promoting a single transition to flowering, as in *Arabidopsis* and *Antirrhinum*.

Another *Arabidopsis* gene, called *TERMINAL FLOWER2 (TFL2)*, also affects the maintenance of inflorescence identity (Larsson *et al.*, 1998). Like *tfl1*, *tfl2* plants flower early and produce terminal floral structures at the apex of the inflorescence. In addition, *tfl2* plants have more variable terminal flower structures and reduced photoperiod sensitivity. The *tfl1 tfl2* double mutant was virtually insensitive to day length, unlike the *tfl1* single mutant, suggesting that these two genes function in distinct pathways. The *tfl2* mutant plants also have additional phenotypes, such as reduced leaf size and shorter internodes, suggesting that *TFL2* has a regulatory role more global than *TFL1*, such as that regulating the meristem response to light signals (Larsson *et al.*, 1998).

4.4.3 Interactions between *LFY*, *AP1 (CAL)* and *TFL1*

LFY, *AP1* and *CAL* all promote floral meristem identity, but appear to do this through at least two pathways. Results from mutant studies, sequence comparison and expression analysis all support the idea that *AP1* and *CAL* have redundant functions (Irish and Sussex, 1990; Mandel *et al.*, 1992; Bowman *et al.*, 1993; Schultz and Haughn, 1993). In contrast, *LFY* and *AP1 (CAL)* appear to function in parallel pathways, as suggested by their mutant phenotypes and expression patterns. For example, the *lfy ap1* double mutant has a much more severe phenotype than *lfy* or *ap1* single mutants (Bowman *et al.*, 1993; Weigel and Meyerowitz, 1993). The *lfy ap1 cal* triple mutant has an even more complete transformation of flowers into shoots, indicating that *LFY*, *AP1* and *CAL* act together to promote the transition from vegetative development to flower meristem (Bowman *et al.*, 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). On the other hand, the *lfy cal* double mutant is similar to *lfy* single mutant, confirming the earlier conclusions that *lfy* is functionally different from *ap1* and that *cal* is a weaker mutant than *ap1*.

The *LFY* gene is expressed earlier than the *AP1* in the wild-type lateral meristems after floral induction (Mandel *et al.*, 1992; Weigel *et al.*, 1992; Blazquez *et al.*, 1997). Furthermore, early flowering and shoot-to-flowering transformation due to constitutive *AP1* expression are not significantly affected by *lfy* mutations, whereas *ap1* mutations suppress the 35S-*LFY* phenotypes (Mandel and Yanofsky, 1995a; Weigel and Nilsson, 1995; Liljegren *et al.*, 1999). These results suggest that *AP1* act downstream of *LFY*. This is further supported by

the observation that the expression of *API* is slightly delayed as well as reduced in the *lfy* mutant (Ruiz-Garcia *et al.*, 1997; Liljegren *et al.*, 1999; Ratcliffe *et al.*, 1999). *LFY* can bind to a putative *API* promoter element *in vitro* and its ectopic constitutive expression causes precocious *API* expression (Parcy *et al.*, 1998), further suggesting that the *API* gene is a transcriptional target of *LFY*. Taking advantage of the technique of steroid-inducible activation of *LFY*, Wagner *et al.* (1999) demonstrated that *LFY* can directly activate *API* transcription.

Interestingly, *API* and *CAL* also positively regulate *LFY* expression (Bowman *et al.*, 1993; Liljegren *et al.*, 1999). In 35S-*API* plants, *LFY* expression was observed to be present at a higher than normal level. Although the level of *LFY* expression appears unchanged in *ap1* single floral meristems, it is dramatically reduced in the meristems of the *ap1 cal* double mutant. Further evidence for a positive interaction between *LFY* and *API* came from the analysis of plants carrying both 35S-*LFY* and 35S-*API* constructs, which flowered much earlier under short days than plants constitutively expressing either gene alone (Liljegren *et al.*, 1999). However, it is still not known whether the regulation of *LFY* expression by *API* and *CAL* is direct.

The maintenance of inflorescence meristem identity by *TFL1* appears to be mediated, at least in part, through interactions with *LFY* and *API*. Both genes are ectopically expressed in the *tfl1* mutant in meristems that would normally develop as inflorescence meristems (Weigel *et al.*, 1992; Bowman *et al.*, 1993; Gustafson-Brown *et al.*, 1994; Bradley *et al.*, 1997). Therefore, *TFL1* represses *LFY* and *API* expression at the inflorescence apex. Recent studies using 35S-*LFY* and 35S-*API* transgenic plants indicate that *LFY* and *API* can also repress *TFL1* expression (Liljegren *et al.*, 1999; Ratcliffe *et al.*, 1999). However, constitutive *TFL1* expression from the 35S promoter can reduce the effect of constitutive *LFY* or *API* expression (Ratcliffe *et al.*, 1999). Therefore, *TFL1* acts in the inflorescence meristem to delay the activation of *LFY* and *API*, thereby maintaining the inflorescence meristem identity, consistent with models of *TFL1* function based on mutant analysis. The effect of the 35S-*TFL1* transgene on 35S-*LFY* and 35S-*API* transgenes also suggests that it can prevent *LFY* and *API* from acting in the floral meristem.

Although the *Antirrhinum* *CEN* gene is the homolog of *TFL1* (Bradley *et al.*, 1996, 1997), its interaction with *FLO* (the *LFY* homolog), is not the same as that between *TFL1* and *LFY*. Whereas there is a mutual repression of expression between *TFL1* and *LFY*, as discussed above, the expression of *CEN* is slightly later than that of *FLO*, and is dependent on *FLO* for its initial expression (Bradley *et al.*, 1996). Subsequently, *CEN* represses *FLO* expression at the center of the inflorescence meristem, similar to the *TFL1* function. Another difference is found with the tomato *CEN/TFL1* homolog *SP*, which is expressed in inflorescence and floral meristems and co-expressed with the tomato *LFY* homolog (Pnueli *et al.*, 1998). Therefore, in tomato, these two genes do not antagonize each other at the mRNA level, unlike the relationship between *TFL1* and *LFY*.

In addition to the control of gene expression, *LFY* activity can move to adjacent cells and act non-autonomously between cell layers and over considerable distance within cell layers, as detected by mosaic studies (Sessions *et al.*, 2000). This may be similar but not identical to *FLO*, which is non-autonomous between layers but exhibits some cell autonomy within cell layers (Carpenter and Coen, 1995). Therefore, the control of *LFY/FLO* activity at the level of protein/RNA cell-cell movement can also affect the domain of the gene activity. For example, *LFY* can potentially activate target gene expression even in cells that have low levels of *LFY* mRNA.

4.4.4 *Genes controlling floral meristem determinacy*

In *Arabidopsis* and many other plants, the floral meristem differs from the inflorescence meristem in that it is determinate, producing only a limited number of floral organs. This determinacy of floral meristems requires the floral regulatory gene, *AGAMOUS* (*AG*) (Bowman *et al.*, 1989, 1991; Yanofsky *et al.*, 1990; Mizukami and Ma, 1997). In *ag* mutants, floral meristems fail to acquire determinacy, producing reiterative 'double flowers' (Bowman *et al.*, 1989). Like *AG*, the *Antirrhinum* homolog, *PLENA*, is also required for floral meristem determinacy (Bradley *et al.*, 1993). *AG* homologs have been isolated from many plants, and they generally have conserved functions (e.g., Kempin *et al.*, 1993; see review in Ma, 1994; Pnueli *et al.*, 1994; Ma and dePamphilis, 2000). However, in maize, the functions of duplicated *AG* homologs appear to have diverged, with one controlling determinacy, while the other functions in the specification of floral organ identity (Mena *et al.*, 1996).

A determinacy function for *AG* in *Arabidopsis* is suggested by the reversion of the floral meristem to an inflorescence meristem in *ag* mutants in which a long-day-dependent flowering stimulus is removed (Okamuro *et al.*, 1996; Mizukami and Ma, 1997). The floral reversion was also observed in the *ag co* double mutant, consistent with the fact that *CO* is required for the long-day flowering pathway (Mizukami and Ma, 1997). Therefore, the floral meristem specified by *LFY* and *API* is still indeterminate, and *AG* is required to ensure that the floral meristem acquires its determinacy. The floral reversion phenotype of the *ag* mutant is suppressed by exogenous GA and the *spy* mutation, as well as by *hyl*, which blocks phytochrome functions (Okamuro *et al.*, 1996). Therefore, the GA pathway seems to activate gene(s) that can prevent floral reversion. In fact, *LFY* (+/–) plants also display floral reversion in a photoperiod sensitive and GA-suppressible manner, suggesting that the level of *LFY* activity may be critical for maintaining floral meristem identity, especially in the absence of *AG* (Okamuro *et al.*, 1996).

AG also functions in the specification of reproductive organ identity, representing the C function of the ABC model (Bowman *et al.*, 1989; Yanofsky *et al.*, 1990; Coen and Meyerowitz, 1991; Mizukami and Ma, 1992; Ma, 1994).

Genetic and molecular studies suggest that the *AG* functions in controlling meristem determinacy and floral organ identity can be separated (Mizukami and Ma, 1995; Sieburth *et al.*, 1995). *AG* encodes a MADS-box transcription factor (Yanofsky *et al.*, 1990). The ectopic *AG* expression using a 35S-*AG* fusion was found to cause terminal flower formation and early flowering. Furthermore, ectopic *AG* function promotes determinate floral fate in the *lfy* and *ap1* mutants (Mizukami and Ma, 1997), supporting the idea that *AG* is downstream of *LFY* and *API* (Weigel and Meyerowitz, 1993).

The *AG* protein was shown to interact physically with other *Arabidopsis* MADS-domain proteins, *AGL2*, *AGL4* and *AGL9*, *in vitro* and in yeast two-hybrid assays (Huang *et al.*, 1996; Fan *et al.*, 1997). The *AGL2*, *AGL4* and *AGL9* genes are also expressed in the floral meristem with temporal and spatial patterns that overlap with that of *AG* (Flanagan and Ma, 1994; Savidge *et al.*, 1995; Mandel and Yanofsky, 1998). Therefore, the interaction between *AG* and these *AGL* proteins may be important for normal flower development. Co-suppression analysis of a *Petunia* gene, *fbp2*, a putative homolog of *AGL2* and *AGL4*, resulted in floral meristem and floral organ defects (Angenent *et al.*, 1994). It has recently been reported that although single mutations in each of the *AGL2*, *AGL4* and *AGL9* genes (renamed as *SEPALATA1*, 2, 3 or *SEP1*, 2, 3) cause no obvious phenotypes, the triple mutant lacking all three gene functions produces flowers with only sepals and indeterminacy in the floral meristem (Pelaz *et al.*, 2001). This phenotype is similar to that of the *ag ap3* double mutant lacking both B and C functions, indicating that the *SEP* genes together are required for floral meristem determinacy and floral organ identity.

In addition to *AG* and *SEP* genes, the *SUPERMAN* (*SUP*, also called *FLO10*) gene also contributes to floral determinacy. Flowers of *sup* mutants produce more than six stamens and display reduced carpel size (Schultz *et al.*, 1991; Bowman *et al.*, 1992). Furthermore, the stamens are arranged in additional whorls in the *sup* mutant flowers, indicating a defect in floral determinacy. The *SUP* gene encodes a C2H2-type zinc finger protein and is expressed at the boundary of regions of the floral meristem for the third and fourth whorls of the flower (Sakai *et al.*, 1995). Therefore, *SUP* appears to regulate cell proliferation by maintaining the boundary between the third and fourth whorl, within the domain of *AG* action. Further analyses have indicated that *LFY* regulates the initiation of *SUP* expression; moreover, the floral homeotic genes *AP3*, *PI* and *AG* act to maintain normal levels of later *SUP* expression, but they are not essential for *SUP* expression in flowers at later stages of development (Sakai *et al.*, 2000).

Another gene that regulates floral meristem determinacy is *CARPEL FACTORY* (*CAF*) (Jacobsen *et al.*, 1999). The *caf* mutant produces flowers with near normal numbers of sepals, petals and stamens in the first three whorls, respectively, but one or two additional whorls of stamens interior to whorl three. Occasionally, multiple whorls of stamens are also produced. The *caf* flowers also have reduced and defective carpel development. The *caf* mutant also exhibits

both leaf and floral organ defects. The floral meristem determinacy defects of *caf* are similar to those of the *sup* mutants discussed above. Interestingly, the degree of indeterminacy is greatly enhanced in the *sup caf* double mutant compared with the single mutant, suggesting that they are partially redundant in this regulation. In addition, the *caf clv* double mutants also show dramatic enhancement of floral meristem over-proliferation, suggesting that *CAF* is redundant with *CLV* in controlling floral meristem cell proliferation.

CAF encodes a protein with an N-terminal DEXH/DEAD-box type helicase and C-terminal RNase III-like domains (Jacobsen *et al.*, 1999). Two transcripts were detected, one corresponds to the full-length mRNA, and the other contains only the RNase III-like regions. Further analysis indicates that *CAF* is expressed non-specifically in the SAM and floral meristems, leaves and floral organs. It has recently been demonstrated that a similar *Drosophila* nuclease, Dicer, is capable of catalyzing the initial step of the double-stranded RNA interference (RNAi) process (Bernstein *et al.*, 2001), suggesting that *CAF* may have a similar role in controlling gene activity by an RNAi-like process.

4.4.5 Other genes affecting floral meristem development

Several additional genes have been identified which contribute to *Arabidopsis* floral meristem development (table 4.2). It was found that *apetala2* (*ap2*) mutations affect the floral meristem identity defects of *ap1* (Irish and Sussex, 1990; Bowman *et al.*, 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). Plants with only an *ap2* mutation, however, do not have floral meristem defects under flower-inducing growth conditions. Interestingly, the *ap2-1* mutant flower produces axillary flowers under short days; this floral meristem defect is suppressed by the *hyl* mutation that is defective in the synthesis of phytochrome chromophore (Okamuro *et al.*, 1997). In addition, exogenous GA and the *spy* mutations also suppress the floral meristem defects of the *ap2-1* mutant. Moreover, when *AP2* is introduced into *Petunia*, it seems to promote the transition from vegetative to reproductive development, consistent with a role in floral meristem identity (Maes *et al.*, 1999). *AP2* encodes a putative transcription factor that contains two repeats (the *AP2* repeats) that are present in many plant regulatory proteins (Jofuku *et al.*, 1994; Weigel, 1995). In maize, an *AP2*-like gene, *indeterminate spikelet 1*, is involved in the control of the maize spikelet meristem fate (Chuck *et al.*, 1998).

Another gene affecting floral meristem identity is the *UNUSUAL FLORAL ORGAN* (*UFO*) gene. At many positions along the floral stem, *ufo* mutants produce a filament instead of a flower (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995), indicating a defect in floral meristem function. Flowers of *ufo* mutants also have floral organ defects, suggesting that *UFO* plays a critical role in regulating floral homeotic genes (see section 4.7). The *Antirrhinum fimbriata* (*fim*) mutant also has abnormal floral organs that are similar to those

in *ufo* flowers. The cloning of *FIM* by transposon tagging enabled subsequent cloning of *UFO* by using *FIM* as a probe (Simon *et al.*, 1994; Ingram *et al.*, 1995). *FIM* and *UFO* appear to be single-copy homologous genes with no close counterparts in non-plant organisms. Molecular studies offer some clues to the function of the genes by revealing the presence of an F-box, a motif that has been shown to be involved in regulating proteolysis in yeast (Skowrya *et al.*, 1997), and showing that the *FIM* and *UFO* proteins interact with plant homolog of the yeast *SKP1* protein (Ingram *et al.*, 1997; Samach *et al.*, 1999; Zhao *et al.*, 1999). The *SKP1* gene is essential for mitosis and regulates key proteins controlling cell cycle and transcription in yeast (Willems *et al.*, 1999), suggesting that *FIM* and *UFO* may regulate cell division in the floral meristem (see section 4.6 for further discussion).

Unusual filamentous structures are also produced instead of flowers in mutants of the *FILAMENTOUS FLOWER (FIL)* gene (Chen *et al.*, 1999; Sawa *et al.*, 1999a, 1999b). These structures are likely to correspond to aborted flowers given high levels of *API* expression. Furthermore, *fil ap1* and *fil lfy* double mutants have more severe meristem defects, and *API* expression is reduced in *fil lfy* mutant filamentous structures, suggesting that *FIL* contributes to a positive regulation of *API* expression. The *FIL* gene encodes a protein with zinc finger and HMG-related domains (Sawa *et al.*, 1999b). *FIL* is expressed at the abaxial side of lateral organs, suggesting that *FIL* affects meristem development indirectly.

The presence of filamentous structures in *ufo* and *fil* mutants suggests that they indicate a failure in floral meristem development (Levin *et al.*, 1998). This view is further strengthened by the observation that double mutants carrying one of a group of mutations, such as *ufo* or *lfy*, and one of another group of mutations, such as *fil*, *clv1* and *clv3*, produce filamentous structures in place of most flowers. These groups of mutations affect different aspects of flower development and are components of different genetic pathways. Therefore, the formation of filamentous structures may represent a general loss of flower-promoting activities. Using this feature of enhancement of *ufo* phenotypes, three new genes were identified, *FFO1*, *FFO2* and *FFO3* (Levin *et al.*, 1998). In addition to causing the production of many filamentous structures when combined with *ufo*, these *ffo* mutations also cause floral organ fusion defects in single mutants.

4.5 Regulation of meristem identity genes

Given that flowering time genes regulate the formation of reproductive meristems, the ultimate functions of these genes must be to regulate meristem identity genes. In *Arabidopsis* and *Antirrhinum*, in which the inflorescence is indeterminate, several genes promote the floral meristem identity, while at the

same time, *TFL1/CEN* have an opposing activity that promotes maintenance of inflorescence identity (table 4.2). Therefore, flowering time pathways must be able to regulate both kinds of genes. This section discusses our current understanding of the regulation of three central meristem identity genes, *LFY*, *API* and *TFL1*.

4.5.1 Regulation of *LFY* expression

LFY is a key floral meristem gene and its regulation is critical to the control of flower development. In the wild-type, *LFY* expression is rapidly elevated under long days and slowly increased under short days (Blazquez *et al.*, 1997). *LFY* was shown to be a target of activation by the long-day pathway by using transgenic plants carrying an inducible *CO-GR* (glucocorticoid receptor) fusion construct (Simon *et al.*, 1996). *LFY* expression was activated quickly following the induction of *CO* function, suggesting a direct regulation. The *LD* gene also has a positive effect on *LFY* expression in the *ap1 cal* background (Aukerman *et al.*, 1999). In addition, analysis of double mutants and the effects of constitutive *LFY* expression in *fca* mutant background supports the idea of a positive regulation of *LFY* by the autonomous pathway (Nilsson *et al.*, 1998; Page *et al.*, 1999). Finally, GA can accelerate the increase of *LFY* expression under short days, suggesting that the GA pathway also positively regulates *LFY* (Blazquez *et al.*, 1998). Therefore, *LFY* is an ultimate target of four flowering pathways.

To test whether different flowering pathways converge before or at the *LFY* promoter, discrete regions of the *LFY* promoter were analyzed for responsiveness to different flower-promoting activities using promoter fusions (Blazquez and Weigel, 2000). Results indicated that the GA pathway acts via *cis* elements that are separate from those responsive to the long-day pathway. Furthermore, analysis of the effect of the *phyB* mutation on *LFY* expression indicated that PHYB represses flowering via a pathway separate from the long-day pathway, and is also independent of the GA pathway (Blazquez and Weigel, 1999). Therefore, the regulation of *LFY* transcription allows the integration of the long-day and GA pathways, as well as the separate repression by PHYB.

4.5.2 Regulation of *API*

As mentioned in section 4.4.3, *LFY* positively regulates *API* expression (Parcy *et al.*, 1998; Wagner *et al.*, 1999). It was shown that although CO activates *API* expression using the GR inducible system, the activation is much delayed compared with the activation of *LFY* expression (Simon *et al.*, 1996). Therefore, it is likely that activation of *API* expression by *CO* is indirect. Furthermore, genetic studies suggest that two genes, *FT* and *FWA*, act in parallel with *LFY* to regulate the *API* gene (Ruiz-Garcia, 1997). In *ft lfy* and *fwa lfy* double mutants, the expression of *API* is not detectable. This regulation is also supported by

the genetic interaction between *lfy* and *fwa* or *ft* mutations (Ruiz-Garcia, 1997). Because loss-of-function *ft* mutations cause a reduction in *API* expression, *FT* is formally an activator of *API*. However, *FT* may not act directly because it is similar to a phosphatidylethanolamine binding protein, which regulates the MAP kinase signaling cascade (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Yeung *et al.*, 1999). Because the *fwa* mutant is a semi-dominant gain-of-function allele, the reduced *API* expression seen in *fwa* suggests that *FWA* normally negatively regulates *API* expression. Because *FWA* encodes a homeodomain protein, a potential transcription factor (Soppe *et al.*, 2000), it is possible that *FWA* can directly repress *API* transcription.

The *Antirrhinum SQUA* gene is a homolog of *API* (Huijser *et al.*, 1992; Mandel *et al.*, 1992). Molecular studies have identified the *SBP1* and *SBP2* genes that encode proteins that bind to the *SQUA* promoter. The expression of these genes is activated before *SQUA* expression, suggesting that they may play a role in regulating *SQUA* expression (Klein *et al.*, 1996). In *Arabidopsis*, the putative *SBP1* homolog, *SPL3*, has been implicated in the regulation of flowering transition because: *SPL3* is mainly expressed in vegetative, inflorescence and floral meristems, and in organ primordia; and constitutive *SPL3* expression causes early flowering (Cardon *et al.*, 1997). Although the *SPL3* protein can bind to the *API* promoter, the 35S-*SPL3* induced early flowering does not depend on *API* function, suggesting that *SPL3* may regulate *API* as well as other genes.

4.5.3 Regulation of and interaction with *TFL1*

TFL1 is expressed in a small region at the center of the inflorescence meristem (Bradley *et al.*, 1997). Using the inducible *CO-GR* system, it was found that *CO* probably activates *TFL1* expression directly (Simon *et al.*, 1996). Because *CO* also activates *LFY* expression, it seems to be responsible for establishing the balance between *TFL1* and *LFY* expression in the central zone and peripheral zone, respectively. Furthermore, *TFL1* and *FT* negatively interact with each other (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). It was observed that *ft* enhances the phenotype of delayed flowering in 35S-*TFL1* plants, and *tfl1* enhances the early flowering of 35S-*FT* plants. Therefore, these genes probably do not act by eliminating each other's activity, or may not even interact directly with each other. Instead, they might control other downstream genes in opposite directions. One such gene is likely to be *API*. In addition, the effect of 35S-*TFL1* is dramatically reduced in the presence of 35S-*FT*, and *ft* is epistatic to *tfl1*, suggesting that *FT* activity is more important in regulating flowering time.

tfl1 mutants also flower early under short days, suggesting that *TFL1* may mediate regulation of flowering by other pathways (Shannon and Meeks-Wagner, 1991; Alvarez *et al.*, 1992). Analysis of flowering time in *tfl1* and *fca* single mutants and *fca tfl1* double mutants indicates that *fca* is epistatic to *tfl1* (Page *et al.*, 1999). This suggests that *TFL1* somehow negatively regulates *FCA*.

although *FCA* expression is not altered in the *tfl1* mutant (Page *et al.*, 1999). Alternatively, *TFL1* could repress another gene that requires *FCA* for expression or activity. The lack of *FCA* function would prevent that gene from functioning, thereby removing the effect of *tfl1* mutation. In other words, *TFL1* could repress genes downstream of the autonomous pathway. As discussed in section 4.4.3, *TFL1* represses *LFY* and *API*, both of which are downstream of the autonomous pathway (Page *et al.*, 1999).

4.6 Control of meristem size and floral organ number

4.6.1 Genes promoting meristem genesis and maintenance

The shoot apical meristem (SAM) has a central zone (CZ), where cells are in an undifferentiated state, and a peripheral zone (PZ), where cells rapidly divide to form organs (Steeves and Sussex, 1989; Bowman and Eshed, 2000). A number of genes have been identified and characterized that are involved in the regulation of SAM development in *Arabidopsis* (table 4.2). One group of genes, including *SHOOT MERISTEMLESS* (*STM*; Barton and Poethig, 1993; Granger *et al.*, 1996; Long *et al.*, 1996; Evans and Barton, 1997) and *WUSCHEL* (*WUS*; Mayer *et al.*, 1998), is required for the initiation and maintenance of the SAM. Another group of genes, including *CLAVATA1* (*CLV1*; Clark *et al.*, 1993, 1997), *CLAVATA2* (*CLV2*; Kayes and Clark, 1998; Jeong *et al.*, 1999) and *CLAVATA3* (*CLV3*; Clark *et al.*, 1995; Fletcher *et al.*, 1999), functions to limit the size and to maintain the integrity of the SAMs. These genes are discussed in detail in chapter 2 of this volume.

In addition to the *STM* and the *CLAVATA* genes, the *WIGGUM* (*WIG*) gene also regulates cell proliferation in the floral apical meristem, as suggested by the increase in petal and sepal number in the mutant (Running *et al.*, 1998). Furthermore, *wig* mutations can enhance *clv3* phenotypes, resulting in abnormally large SAMs (Running *et al.*, 1998; Ziegelhoffer *et al.*, 2000). *WIG* encodes a farnesyltransferase β -subunit and is identical to the *ERA1* gene (Ziegelhoffer *et al.*, 2000). The importance of farnesylation in flower development is further supported by the discovery that the AP1 protein is farnesylated *in vivo* and *in vitro*. In addition, mutant forms of AP1 that cannot be farnesylated fail to induce the expected phenotypes of constitutive *API* expression (Yalovsky *et al.*, 2000). Furthermore, the *era1-2* mutation can suppress the phenotypes of constitutive *API* expression, indicating that *ERA/WIG* is involved in this form of post-translational regulation of AP1 (Yalovsky *et al.*, 2000).

Mutations in the *MGOUN1* (*MGO1*) and *MGOUN2* (*MGO2*) genes cause the formation of a large SAM and fasciation of the inflorescence stem (Laufs *et al.*, 1998). The *mgo1* mutation enhances the organ initiation defect of the weak *stm-2* mutant but not the strong allele *stm-5*. In addition, the enlarged meristems in *mgo1* and *mgo2* undergo continuous fragmentation, resulting in

multiple branching, unlike the *clv1* and *clv3* phenotypes. Double mutant studies indicate that *MGO1/MGO2* and *CLV1/CLV3* effects are additive, suggesting that *MGO1* and *MGO2* are parts of a different pathway from that of *CLV1* and *CLV3*.

4.6.2 Control of floral organ number by meristem genes and meristem identity genes

Floral organ number is controlled by a number of genes, including those that regulate SAM development, meristem identity and floral organ identity. Genes controlling SAM development and size also regulate floral meristem size, thereby affecting floral organ number. In particular, plants with different alleles of *STM* (*stm-2*, *stm-5*, *stm-6*) have greatly reduced floral meristems, leading to dramatic loss of floral organs (Clark *et al.*, 1996; Long and Barton, 2000). Although the organ number in the first whorl of flowers in these mutants is nearly normal, the organ number interior to whorl one is greatly reduced, and the prematurely terminated flowers have no central organs (Endrizzi *et al.*, 1996). Like *STM*, *WUS* also controls the floral organ number. Although the *wus* mutant flowers produce the normal number of organs in the two outer whorls, they terminate in a single stamen at the center (Laux *et al.*, 1996). Both *STM* and *WUS* affect floral organ number indirectly by regulating floral meristem size, because they are not expressed in regions where the floral organ primordia are initiated (Long *et al.*, 1996; Mayer *et al.*, 1998; Long and Barton, 2000).

In addition, the *clv* mutants have increased meristem size and greater numbers of all types of floral organs (Clark *et al.*, 1993, 1996; Kayes and Clark, 1998). The severe *clv1* and *clv3* single mutants and *clv1 clv3* double mutants have phenotypes with similar organ number. The *ag-2* mutant also produces extra whorls of floral organs and it enhances the *clv1* phenotype in floral organ number; however, the *ap1 clv1* double mutant has fewer floral organs than the *clv1* single mutant (Clark *et al.*, 1993). Also, the *wig* mutation causes striking increases in floral organ number, especially of sepals and petals (Running *et al.*, 1998; Ziegelhoffer *et al.*, 2000).

The meristem identity genes also affect the regulation of floral organ number. In the *lfy-6* mutant, a strong allele, flowers have a slight reduction of floral organ number, but the weak mutant *lfy-5* has a greater reduction of floral organ number compared to the *lfy-6*, especially of petals (Weigel *et al.*, 1992). The *ap1-1* flower consists of a normal number of sepal-like bracts in the first whorl, frequently normal numbers of stamens and carpels, but usually no petals in the second whorl (Irish and Sussex, 1990; Mandel *et al.*, 1992; Bowman *et al.*, 1993). Moreover, the floral organ identity genes, or so-called ABC genes, also contribute to the control of floral organ number. Mutations in the B function genes, *AP3* and *PI*, cause a slight reduction of floral organ number interior to the first whorl (Bowman *et al.*, 1989). The A and C function genes have an even

more dramatic effect on floral organ number. For example, severe mutations in the A function gene *AP2* (e.g. *ap2-2*) cause the loss of some first and all second whorl organs in most flowers (Kunst *et al.*, 1989, Bowman *et al.*, 1991). The C function gene, *AG*, controls the floral determinacy, limiting the formation of internal flowers at the center of floral meristems. The strong loss-of-function allele, *ag-1*, leads to dramatic increases in the number of sepals and petals (Bowman *et al.*, 1989, Yanofsky *et al.*, 1990).

4.6.3 The *PERIANTHIA* gene controls the tetramerous floral pattern

The *PERIANTHIA* (*PAN*) gene has a specific phenotype in regulating floral organ numbers (Running and Meyerowitz, 1996). In *pan* mutants, both sepal and petal numbers are converted from four to five and stamen number is altered from six to five, whereas the number of carpels is unaffected. Genetic analysis indicates that mutations in meristem identity genes (*LFY* and *API*) are epistatic to *pan* mutations, suggesting that *PAN* may be downstream of *LFY* and *API* (Running and Meyerowitz, 1996). Similar mutant analysis suggests that *PAN* is independent of floral organ identity genes (*AP2*, *AP3*, *PI* and *AG*) and SAM genes (*CLV1*, *CLV2*, *CLV3* and *WIG*) (Running and Meyerowitz, 1996; Running *et al.*, 1998). *PAN* could change the floral organ number by controlling the position of space for cell division that leads to organ initiation, and would thus contrast with *CLV* genes, which affect organ number indirectly via increases in the size of the floral meristem. *PAN* encodes a member of the basic region/leucine zipper class of transcription factors and is expressed in SAMs, floral meristems and each whorl of organ primordia. Consistent with genetic studies, *PAN* expression is not affected by genes controlling floral meristem size or floral organ number (Chuang *et al.*, 1999). The fact that *PAN* expression is also not altered in *lfy*, *ap1* or *ap2* mutants could be explained if genes affected by these mutations have redundant functions in regulating *PAN* expression or if *PAN* activity requires a gene that is regulated by the meristem identity genes.

4.6.4 Other genes affecting floral organ number

Several others genes also regulate floral organ number, as well as having other functions in flower development. The *aintegumenta* (*ant*) mutant shows reduced number and size of floral organs as well as defects in the initiation and growth of the integument during development of the ovule (Elliott *et al.*, 1996; Klucher *et al.*, 1996). The *ANT* gene encodes a putative transcriptional factor containing AP2-repeats and is expressed in young flowers, ovule primordia and all lateral shoot organs (Elliott *et al.*, 1996; Klucher *et al.*, 1996; Nole-Wilson and Krizek, 2000), suggesting that *ANT* functions in organ primordium initiation and shoot development. Further studies have led to the proposal that *ANT* may

coordinate cell growth by maintaining competence for meristematic growth during organogenesis (Mizukami and Fischer, 2000). Mutations in the *TOUSLED* (*TSL*) gene also cause the loss of floral organs. It has been proposed that this gene, which encodes a protein kinase, regulates the initiation of floral organ primordia (Roe *et al.*, 1993, 1997a, 1997b).

The *LEUNIG* (*LUG*), *TSO1*, *ETTIN* (*ETT*), *PETAL LOSS* (*PTL*) and *FIL* genes also contribute to the regulation of floral organ number (Liu and Meyerowitz, 1995; Liu *et al.*, 1997; Sessions *et al.*, 1997; Chen *et al.*, 1999; Griffith *et al.*, 1999; Sawa *et al.*, 1999a). In *lug* mutants, the flowers produce a reduced numbers of petals and stamens (Liu and Meyerowitz, 1995). The *tso* mutant flowers produce slightly more sepals, usually five to six (Liu *et al.*, 1997). In *ett* mutant flowers, the number of sepals and petals is increased but the number of stamens is decreased (Sessions *et al.*, 1997). Both *LUG* and *ETT* encode putative transcriptional regulators, with *ETT* similar to proteins that bind to auxin responsive promoter elements and *LUG* similar to transcription co-repressors that contain WD-repeats (Sessions *et al.*, 1997; Conner and Liu, 2000). The *TSO1* protein contains cysteine-rich repeats (Song *et al.*, 2000) and is similar to the *Drosophila* Enhancer-of-zeste protein, a member of the Polycomb group proteins regulating chromatin structure and gene expression (Jones and Gelbart, 1993).

Mutations of the *UFO* gene result in the loss of floral organs in whorls two and three and additional variable floral organ defects (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). The *UFO* gene is a homolog of the *Antirrhinum* *FIM* gene and encodes a protein with a putative F-box (Ingram *et al.*, 1995; Samach *et al.*, 1999). F-box proteins are found to be part of the SCF ubiquitin ligase, consisting of SKP1, CULLIN in addition to an F-box protein (Skowyra *et al.*, 1997). The SCF complexes regulate cell division and other cellular processes by mediating the degradation of regulatory proteins (Peters, 1998).

A mutation in the *ASK1* gene, a homolog of SKP1, was found by transposon mutagenesis and named *ask1-1* (Yang *et al.*, 1999). The *ask1-1* mutant flower exhibits a reduction in both number and size of petals, as well as the length of filaments (Zhao *et al.*, 1999), which are similar to some of the changes observed in *ufo* mutants. Because *ASK1* is a homolog of *SKP1*, *ASK1* might interact with the F-box *UFO* protein to form a complex. Indeed, double mutant analysis of *ask1-1* and *ufo* suggest that these two genes interact genetically (Zhao *et al.*, 1999). Furthermore, *UFO* and *ASK1* proteins can interact in a yeast two-hybrid experiment (Samach *et al.*, 1999). Similarly, the F-box protein *FIM* also interacts with SKP1 homologs in *Antirrhinum* (Ingram *et al.*, 1997). Therefore, *UFO* and *FIM* probably form SCF complexes with homologs of SKP1 and CULLIN to regulate protein degradation. The *UFO* expression pattern and the *ufo* and *ask1* mutant phenotypes suggest that *UFO* and *ASK1* affect cell division, most probably in regions of the floral meristem responsible for whorls three and four

(Ingram *et al.*, 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Samach *et al.*, 1999; Zhao *et al.*, 1999).

The fact that many genes affect floral organ number suggests that there are multiple pathways regulating floral meristem activity that lead to stereotypical patterns of floral organ numbers. Floral organ number could be controlled by regulating the overall proliferation of cells in floral meristem (SAM genes), or by regulating the relative amount of cell division in specific whorls or regions in the floral meristem (e.g. *UFO*, *PAN*). The fact that floral homeotic genes (e.g. *AP2* and *AP3*) in *Arabidopsis* also affect floral organ numbers suggests that these genes also regulate floral organ primordia initiation, possibly by affecting cell division (Bowman *et al.*, 1991; Jenik and Irish, 2000; Sakai *et al.*, 2000). In addition, the effect of meristem identity genes is, at least in part, regulating organ identity genes (see section 4.7). The reduction in organ number is due to a failure of organ primordia formation, before the specification of floral organ identity. The fact that homeotic genes, such as *AP3* and *AG*, are expressed relatively late in the floral meristem after the initiation of sepal primordia but before that of other organs, indicates that the floral meristem is not determinate at this time and can still respond to these gene functions. Furthermore, molecular and biochemical studies are necessary to understand how these genes act at the molecular level to control floral organ number.

4.7 Interactions among and regulation of floral organ identity genes

The function of the floral meristem is to generate floral organs: sepals, petals, stamens and carpels. The four types of floral organs are specified by the ABC functions representing floral homeotic genes in *Arabidopsis* and *Antirrhinum* (Coen and Meyerowitz, 1991; Meyerowitz *et al.*, 1991; Ma, 1994; Weigel and Meyerowitz, 1994; Ma and dePamphilis, 2000). The homologs of these genes have been characterized in a number of eudicotyledonous plants and a few monocotyledonous plants (Veit *et al.*, 1993; Ma, 1994; Kramer and Irish, 1999; Ma and dePamphilis, 2000). In *Arabidopsis*, A function requires the *AP1* and *AP2* genes, B function requires *AP3* and *PI* genes, and C function requires *AG* gene. The interactions between *LFY* and *AP1* have already been discussed. This section describes recent progress in the identification of additional genes required for controlling floral organ identity in *Arabidopsis*, the demonstration on their interactions with previously discovered ABC genes, and the understanding of regulation of B and C function genes by meristem identity genes, as well as other relevant genetic interactions.

4.7.1 The SEP genes and their role in controlling floral organ identity

According to the ABC model, different functions, either alone or in combination, can specify particular floral organ identities (Coen and Meyerowitz, 1991;

Meyerowitz *et al.*, 1991; Ma, 1994; Weigel and Meyerowitz, 1994). All of the ABC genes, except *AP2*, are members of the MADS-box gene family, and in some cases their protein products have been found to interact *in vitro* (Riechmann and Meyerowitz, 1997). For example, the B function proteins from *Antirrhinum* (DEF and GLO) and *Arabidopsis* (*AP3* and *PI*) bind to DNA as heterodimers, respectively. However, it is not known how the *AP3* and *PI* genes interact with the A function genes *AP1* (and *AP2*) to specify petal identity or the C function *AG* gene to control stamen identity. In fact, the *AP3* or *PI* proteins cannot bind to DNA as heterodimers with *AP1* or *AG* (Riechmann *et al.*, 1996). Furthermore, ectopic expression of B function or C function genes is able to convert floral organ identities within the flower, but is not sufficient to convert leaf into floral organs (Mizukami and Ma, 1992; Krizek and Meyerowitz, 1996).

These results suggest that additional floral specific genes are required for controlling floral organ identity. As mentioned in section 4.4.4, the *SEP1*, 2 and 3 genes (previously named *AGL2*, 4 and 9; Ma *et al.*, 1991; Mandel and Yanofsky, 1998) have redundant functions in specifying the identity of the inner three whorls of floral organs (Pelaz *et al.*, 2001). The fact that the *sep1,2,3* triple mutant flowers produce only sepals resembling those of *ag ap3* double mutant flowers, indicates that both B and C functions require the *SEP* genes. In fact, co-expression of the *SEP3* gene and A and B function genes (*AP1*, *AP3* and *PI*) is sufficient to convert leaves to petals in transgenic plants (Pelaz *et al.*, 2001). In addition, co-expression of *AP3*, *PI*, *AG* and *SEP3* converts leaves into staminoid organs (Honma and Goto, 2001). Therefore, A and B function plus a *SEP* gene are sufficient for petal identity in the leaf background, and B and C functions plus a *SEP* gene are sufficient for stamen identity.

The genetic interactions between the *SEP* genes and the B and C function genes are also supported by the findings that these genes represented the vast majority of the clones isolated when a yeast two-hybrid selection for AG-interacting proteins was performed (Fan *et al.*, 1997). Furthermore, *SEP3* can also interact with *AP1* and with a mixture of *AP3/PI* proteins using both yeast two-hybrid and *in vitro* co-immunoprecipitation assays (Honma and Goto, 2001). Furthermore, *SEP3* can facilitate the formation of a complex that contains AG, *AP3/PI* and *SEP3* (Honma and Goto, 2001). Therefore, the molecular basis for part of the ABC model may be that the *AP1/SEP/AP3/PI* and *AP3/PI/SEP/AG* DNA-binding complexes control genes required for petals and stamens, respectively. The *sep* triple mutant phenotype (Pelaz *et al.*, 2000) suggests that an AG/SEP complex could be responsible for carpel identity genes. Using the yeast two-hybrid assay, it was found that AG and SEP proteins interact via the K domain, whereas the AG homodimer and *AP3/PI* heterodimer formation require the MADS-domain and adjacent I region (Mizukami *et al.*, 1996; Riechmann *et al.*, 1996; Fan *et al.*, 1997). Therefore, different proteins in these complexes may interact using different contacts, adding another level of complexity.

4.7.2 Regulation of AP3/PI expression by LFY/UFO/ASK1

In addition to the *AP3* and *PI* genes, *LEAFY* is also required for specifying petal and stamen identity. The *lfy* single mutant flowers show defects in petal and stamen identity and *lfy ap3* and *lfy pi* double mutants are similar to the *lfy* single mutants (Weigel *et al.*, 1992). *AP3* and *PI* expression are reduced to almost undetectable levels in strong *lfy* mutant backgrounds (Weigel and Meyerowitz, 1993). These results suggest that *AP3* and *PI* may be downstream of *LFY*. Furthermore, constitutive expression of *AP3* can rescue the petal and stamen defects in the *lfy* mutant background (Jack *et al.*, 1994), supporting the hypothesis that B function genes are downstream of *LFY*.

However, ectopic expression of *LFY* alone does not cause an expansion of the region of *AP3* expression, suggesting a requirement for additional factor(s). The *ufo* mutants have defects in petal and stamen development, and *AP3* is expressed precociously and ectopically in *35S-UFO* flowers, indicating that *UFO* is an upstream regulator of *AP3* (Lee *et al.*, 1997; Samach *et al.*, 1999). On the other hand, *UFO* loss- and gain-of-function phenotypes are masked in strong *lfy* background, supporting the idea that *UFO* can only function in the presence of *LFY* activity. Moreover, constitutive expression of both *LFY* and *UFO* is sufficient to induce *AP3* expression in vegetative tissues of seedlings (Parcy *et al.*, 1998). Therefore, in contrast to the activation of *API* by *LFY* alone, activation of *AP3* requires both *LFY* and *UFO*. Analyses of both *AP3* and *PI* promoters have identified regions responsive to *LFY* or *UFO* regulation (Hill *et al.*, 1998; Tilly *et al.*, 1998; Honma and Goto, 2000). The fact that *ASK1* and *UFO* interact physically and genetically suggests that *ASK1* may also participate in this regulation (Samach *et al.*, 1999; Zhao *et al.*, 1999). Because *ASK1* and *UFO* are likely subunits of a ubiquitin ligase that regulates protein degradation, it is possible that they facilitate the removal of a transcriptional repressor or co-repressor of the *AP3* and *PI* genes.

4.7.3 Regulation of AG expression by LFY and other genes

The *AG* gene is expressed at the center of the floral meristem and is required for stamen and carpel identities (Bowman *et al.*, 1989, 1991; Yanofsky *et al.*, 1990). Both *lfy* single mutant and *lfy ap1* double mutants exhibit defects in reproductive organ identity, suggesting that *LFY* and *API* may affect *AG* expression or function (Weigel *et al.*, 1992). On the other hand, *lfy ag* double mutants have distinct phenotypes from those of *lfy* single mutants, indicating that *AG* is still active even in the *lfy* null mutant (Weigel *et al.*, 1992). *AG* expression in *lfy* mutants is delayed in the abnormal flowers but later reaches levels similar to that in the wild-type (Weigel and Meyerowitz, 1993). In addition, *AG* RNA is present ectopically in the stem, as well as some later-formed flowers of *lfy-6*, a strong mutant (Weigel and Meyerowitz, 1993). These results suggest that the

effect of *LFY* on *AG* during early flower development may be different from that at a later stage. In plants that express an activated version of *LFY* protein, the *AG* is expressed earlier, ectopically and at higher level than that in the wild-type plants, suggesting that *LFY* is a primary activator of *AG* throughout the flower (Parcy *et al.*, 1998).

Analysis of the *AG* genomic sequence has indicated that proper *AG* expression requires *cis* elements located in a large intron of about three kilobases (Sieburth and Meyerowitz, 1997). Further dissection of this intron has revealed that there are at least two redundant regions containing enhancer elements that mediate regulation by *LFY* (Busch *et al.*, 1999). Further studies have demonstrated that *LFY* can bind to two sites (*AG I* and *AGII*) *in vitro* and that binding to these sites is correlated with *in vivo* activation of gene expression (Busch *et al.*, 1999). Taken together, these results indicate that *LFY* is likely to be a direct transcriptional regulator of *AG*.

Expression of *AG* is also negatively regulated by *AP2*, *LUG* and *CURLY FLOWER (CLF)* (Drews *et al.*, 1991; Liu and Meyerowitz, 1995; Goodrich *et al.*, 1997). *CLF* encodes a protein similar to the *Drosophila* SET-domain containing Polycomb group proteins. Furthermore, the *clf* mutant flowers early (Goodrich *et al.*, 1997); therefore, *CLF* normally acts to delay flowering by repressing flowering genes. As mentioned in section 4.4.5, *AP2* is a putative transcription factor (Jofuku *et al.*, 1994), suggesting the possibility of direct regulation. Because *AP2* alone is not sufficient to repress *AG*, and *LUG* is probably a co-repressor (Jofuku *et al.*, 1994; Conner and Liu, 2000), these two proteins may form a complex for transcriptional repression. Because *LUG* is also expressed throughout the flower, it does not provide the positional information for *AG* repression. Analysis of the large *AG* intron sequences indicates that there are two redundant regions for *AP2* repression. Furthermore, the repression of early, but not late, *AG* expression requires *LFY* function (Bomblies *et al.*, 1999).

4.7.4 Regulation of *AP1* expression by *AG*

AP1 is expressed initially throughout the early floral meristem prior to sepal primordia initiation, where its expression is limited to whorls one and two and excluded from whorls three and four (Mandel *et al.*, 1992; Gustafson-Brown *et al.*, 1994). This expression coincides with the initiation of sepal primordia and the first expression of *AG* (Yanofsky 1990; Drews *et al.*, 1991). The region in which *AG* expression is present coincides with the region from which *AP1* expression is absent (Gustafson-Brown *et al.*, 1994). Therefore, *AG* might be responsible for excluding *AP1* expression from whorls three and four. Indeed, *AP1* RNA was observed in the entire flower of *ag* mutant during stage three and later stages in the flower development. The fact that *AP1* expression domain expands into inner whorls in the absence of *AG* function indicates that *AG* negatively controls *AP1* expression (Gustafson-Brown *et al.*, 1994).

4.8 Conclusion and perspectives

Genetic and molecular analyses in *Arabidopsis* and other plants have uncovered a large number of genes that play important roles in regulating reproductive meristems in terms of the timing of their formation, their identity, size and activity. These studies have already revealed that there are many parallel pathways controlling flowering time. The sequences of many flowering time genes indicate that although transcriptional regulators are important, other regulatory proteins also play critical roles, including photoreceptors, protein kinases, RNA-binding proteins and membrane proteins. Therefore, the complexity of multiple pathways revealed by genetic studies is further magnified by the multitude of biochemical interactions of the gene products. In addition, we are beginning to appreciate how multiple pathways might be integrated to regulate flowering, with recent molecular genetic studies suggesting that it is unlikely that there will be a single all-encompassing integrator of all pathways. Instead, signals from different pathways seem to be combined in a step-wise fashion. For example, *FLC* responds to both the autonomous and vernalization pathways. In turn, *AGL20* is regulated by *FLC* and the long-day pathway. Furthermore, the *LFY* promoter can respond separately to the long-day and GA pathway, suggesting that it too plays a role in this integration.

Multiple parallel pathways also operate in controlling meristem identity and regulating meristem size and floral organ number. Although the molecular activities of *LFY* in regulating expression of several downstream genes have been defined, such an understanding for other meristem identity genes awaits further investigation. In addition, the genetic and molecular interactions among the genes that affect meristem size and floral organ number are generally not known. Because angiosperm species exhibit a great deal of diversity in floral meristem size and organ number, gene functions controlling this aspect of flower development vary significantly among different plants. It is possible that homologous genes have diverged in different plants. The data available on *LFY/FLO* and *AP2* homologs suggest that this might be an important mechanism for evolution of reproductive structures in flowering plants. It is also possible that gene functions might have been gained or lost during evolution. For example, the *CENFL1* homolog might be absent in plants with terminal flowers. In addition, plants with tetramerous flowers may have acquired genes, such as *PAN*.

The success of genetic and molecular studies is a testament of the power of forward genetics; with tools such as activation tagging, forward genetics will continue to make important contributions, allowing the discovery of even redundant genes. Indeed, gene redundancy has been revealed repeatedly. In some cases, different parallel pathways, such as those regulating flowering time, control the same process under different conditions. In other cases, different genes may be similar in sequence and in biochemical function and also affect

the same process, such as *API* and *CAL*, or the *SEP* genes. At the same time, the completion of the *Arabidopsis* genomic sequencing (Arabidopsis Genome Initiative, 2000) and the ongoing development of reverse genetic tools, such as double-stranded RNA interference (Chuang and Meyerowitz, 2000; Smith *et al.*, 2000), will yield ever increasing knowledge about the molecular machinery underlying the mystery of reproductive meristems.

Clearly genetic and molecular studies in *Arabidopsis* and a few other model plants have been extremely informative. On the other hand, as noted at the beginning of this chapter, *Arabidopsis* and *Antirrhinum* represent only one type of inflorescence structure with highly specialized floral patterns. The angiosperm world offers a wondrous collection of inflorescence and floral forms, which must rely on the divergent activities of respective reproductive meristems. Although homologs of some of the genes discussed here have been identified in a limited number of flowering plants, many more discoveries are yet to be made. Molecular genetic studies in plants such as maize have begun to provide direct evidence for both conservation and divergence. Future progress in these areas promises to be very illuminating. Finally, molecular evolutionary analyses using a much wider collection will be extremely important and informative. Although the past decade has been wonderfully exciting, the next will undoubtedly surpass it in both depth and scope.

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5 Axillary meristem development

Vojislava Grbić

5.1 Introduction

Plants display a large range of morphological forms which can be ascribed to two stages of development. During embryogenesis, the primary apical-basal growth axis is established as well as two groups of proliferative cells, the embryonic shoot and the root apical meristems. During postembryonic development, secondary growth axes develop. As the leaves develop on the primary shoot, axillary meristems form in their axils, giving the plant the potential to develop secondary growth axes. The placement of axillary meristem initials, the timing of their activation, their relationship to the primary shoot apical meristem and their developmental fate have a profound influence on the architecture of the mature plant. In this chapter, our current understanding of the molecular mechanisms underlying axillary meristem and branch development is discussed.

5.2 Branching patterns

5.2.1 *Branching types*

Plants exhibit three types of branching pattern: dichotomous, sympodial and monopodial. Dichotomous branching usually initiates by bifurcation, the splitting of the apical meristem into two halves, thus creating two new growth axes out of one (figure 5.1A). The two new meristems are typically equal in size and newly formed branches are of the same hierarchy. Therefore, the morphological characteristics of dichotomous branching in the shoot are the absence of a dominant axis and the occurrence of a sequence of paired branches that are not associated with subtending leaves. During typical sympodial or monopodial growth, branches do not initiate directly from the shoot apical meristem, but instead are initiated from axillary meristems formed in leaf axils. Subsequent development transforms the axillary meristem into a branch. Therefore, during sympodial and monopodial growth, branching is axillary, with the shoot apical meristem initiation pattern combining the ability to create leaf and branch appendages simultaneously. Monopodial and sympodial patterns of shoot development can be distinguished by the relative contribution of the axillary meristems to the main growth axis. If the main axis is derived from the single shoot apical meristem, then it is referred to as monopodial growth

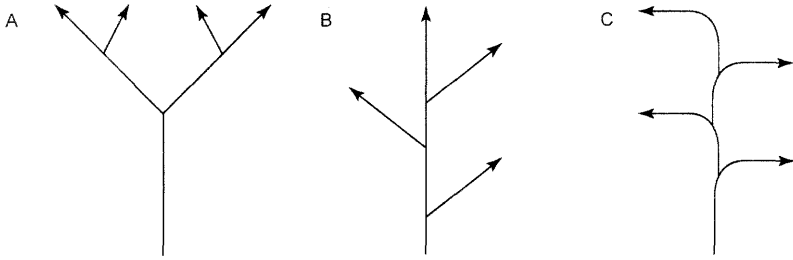


Figure 5.1 Diagrammatic representation of shoots with: A) dichotomous; B) monopodial; and C) sympodial, branching patterns.

(figure 5.1B). However, if the primary shoot apical meristem terminates its development, either by differentiation into a non-meristematic structure or by abortion, and if axillary bud function takes over the further extension of the main axis, then the resulting axis is composed of a linear series of shoot units referred to as a sympodium (figure 5.1C).

5.2.2 Evolution of branching types

Vascular plants have evolved over 470 million years. The fossil reconstruction of extinct vascular plants from the mid-Silurian indicates that they consisted of dichotomizing leafless aerial stems arising from a horizontal rhizomatous system, thus establishing dichotomous branching as an ancestral branching type. However, even the early vascular plants displayed several different branching patterns based on dichotomizing axes. For example, the isotomous branching found in *Rhyniophyta* had daughter branches of the same size and vigour. However, processes of overtopping and reduction lead to the out- or overgrowing of one branch of the dichotomous pair, resulting initially in a pseudo-sympodial type of branching and finally in the pseudo-monopodial type found in *Zosterophyllophyta* and *Trimerophyta* (figure 5.2). *Zosterophyllophyta* evolved into extant *Lycophyta*, which has leaf-like appendages, called microphylls, that are not associated with dichotomously arising branches. *Trimerophyta* exhibit a variety of dichotomy-based branching patterns, but also include *Psilophyton crenulatum*, whose branching appears to be axillary.

Trimerophyta are ancestors to several taxonomic groups that evolved different branching systems: *Psilophyta*, *Sphenophyta*, *Pteridophyta* and *Progymnospermophyta*. *Psilophyta* contains two genera, *Psilotum* and *Tmesipteris*, whose growth habit resembles that of a *Rhyniophytes* since they have leafless dichotomous axes. However, recent phylogenetic analysis suggests that *Psilotidae* are not direct descendants of early vascular plants (Pryer *et al.*, 2001). Their body plan reflects phenotypic simplification that has been derived from the more complex ancestor. The division *Sphenophyta* (horsetails) contains several genera

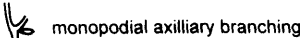


Figure 5.2 Phylogeny of vascular plants highlighting the events leading to the establishment of the branching patterns of living vascular plants (after Stewart, 1964; Stewart and Rothwell, 1993; Taylor and Taylor, 1993; Pryer *et al.*, 2001).

of extinct plants and one genus of living plants, *Equisetum*. Horsetails evolved a monopodial axis that initiates both leaves and branches. However, the branches are not associated with the leaf axil but are produced by the shoot apical meristem, resulting in a growth pattern in which leaves and non-axillary branches alternate. *Pteridophyta* (ferns) display an array of branching types in both living and fossil species, ranging from dichotomous apical branching to axillary. Finally, *Trimerophyta* also gave rise to *Progymnospermophyta*, the lineage that leads to *Angiosperms*. This division contains only extinct species that had pseudo-monopodial (dichotomous) growth, where no axillary buds are produced. Even though *Progymnospermophyta* had non-axillary branching it gave rise to lineages which developed axillary branches independently—these include *Ginkophyta*, *Coniferophyta*, *Cycadophyta*, *Pteridospermophyta* (all extinct) and *Magnoliophyta*.

While flowering plants display a variety of shoot forms based on monopodial and sympodial branching, there are a few species that have dichotomous branching. Dichotomy has been described in dicotyledonous species, such as *Mammillaria* (Boke, 1976) and *Asclepias*, and in a number of monocotyledonous, species including *Chamaedorea* (Fisher, 1974), *Flagellaria* (Tomlinson, 1970), *Nypa* (Tomlinson, 1971) and *Strelitzia* (Fisher, 1975). These species are interspersed with taxa that have sympodial or monopodial branching patterns. The multiple independent losses of mechanisms underlying sympodial or monopodial branching is the most parsimonious way to explain the broad phylogenetic distribution of dichotomous branching, which is regarded as a primitive state. Consistent with this possibility is the mutagen-induced dichotomy in maize, reported by Mouli (1970).

Living vascular plants display a wide array of branching patterns, varying from the ancestral dichotomous, such as in *Lycopods*, *Psilotum* and some higher plants, to monopodial, forming an axis with alternating leaves and branches, as in *Equisetum*, and finally to an axillary branching axis as in ferns, conifers and flowering plants. As we begin to understand the molecular mechanisms underlying axillary branching in model plant organisms, it will be important to determine the conservation of regulatory elements in these phylogenetic groups in order to reconstruct the evolutionary events at the mechanistic level.

5.2.3 Genetic control of branching types

Monopodial and sympodial branching patterns characterize the growth patterns in higher flowering plants. While these two branching types have distinctive characteristics, distinguishing the subtleties of variation between them can require a detailed developmental analysis. Moreover, most sympodially branching plants initially establish a monopodial growth pattern, and then switch to sympodial development only at the transition to the reproductive phase. Finally, some monopodial plants have a subset of lateral branches assuming

the sympodial pattern. This confirms that both monopodial and sympodial branching patterns can exist on a single plant and emphasizes the importance of characterizing their salient differences in order to dissect their underlying molecular mechanisms. *Arabidopsis*, *Antirrhinum* and tobacco are current model plants that form monopodial axes, while *Petunia* and tomato are representatives of sympodially branching species (figure 5.3). During vegetative development, the shoot apical meristems of all these species initiate leaf primordia in whose axils the axillary meristems form. Axillary meristems, when activated, reiterate the developmental pattern of the main shoot. During vegetative development, most species form a monopodial axis. The primary variable in the shoot morphology at this stage is exemplified by *Arabidopsis*, in which the basal internodes fail to elongate, giving rise to the basal rosette of leaves. While the rosette habit has a profound consequence on overall plant morphology, it does not affect the pattern of branching. Transition to reproductive development, however, correlates with the diversification of the shoot forms that are characteristic of each species.

Although the primary shoot apical meristem in *Arabidopsis* and *Antirrhinum* remains indeterminate during reproductive development and continues to initiate primordia on its flank, the pattern of axillary meristem development differs. In *Antirrhinum*, these primordia give rise to leaves and axillary meristems with a floral identity. This initiation pattern results in the formation of flowers subtended by bracts at the topmost part of the shoot (figure 5.3A). In *Arabidopsis*, the reproductive primordia form flowers that are not subtended by bracts (figure 5.3B), suggesting that the basic pattern of reproductive primordia development illustrated by *Antirrhinum* has been modified. Several lines of evidence suggest that the *Arabidopsis* reproductive primordium initially consists of both floral and bract initials, and that bract development is suppressed during later primordium development. First, the existence of meristematic and leaf domains within young reproductive primordia have been demonstrated by the analysis of the expression patterns of the marker genes. The *SHOOTMERISTEMLESS* (*STM*) gene has been used as a marker of the meristematic cell fate, since it has been shown that *STM* is required for shoot meristem formation and maintenance (Barton and Poethig, 1993; Long *et al.*, 1996), while the *AINTEGUMENTA* (*ANT*) gene has been used as a marker for leaf identity, as it is expressed in young organ primordia throughout development (Elliot *et al.*, 1996). During early reproductive primordia development, *STM* and *ANT* mRNA expression domains divide the primordia into two unequal parts: the *STM* (meristematic domain) is expressed in all primordial cells except those within the small domain on the abaxial side of the primordium. The cells that lacked the *STM* expression were expressing the *ANT* gene, indicating the partitioning of the primordium on meristematic and leaf domains (Long and Barton, 2000). A second line of evidence involves the use of *LEAFY* (*LFY*)

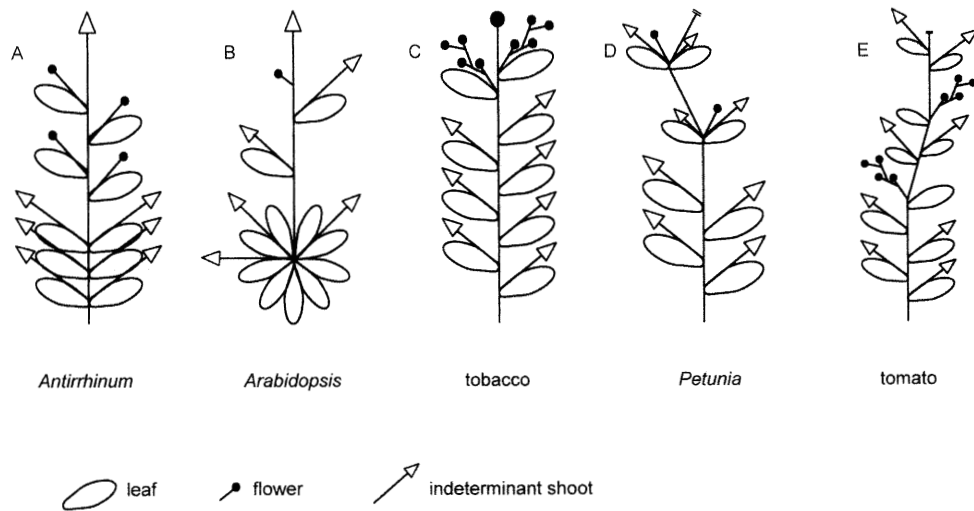


Figure 5.3 Diagrams of various types of plant form. A and B: *Antirrhinum* and *Arabidopsis*, forming an indeterminate monopodial shoot. C: tobacco, forming determinate monopodial shoot. D and E: *Petunia* and tomato, forming sympodial shoot.

promoter (Weigel *et al.*, 1992), fused to a gene encoding the *diphtheria toxin* A chain, which leads to the selective ablation of reproductive primordia. The resulting transgenic plants formed shoots in which flowers were replaced by bracts, suggesting that the induction of floral development normally suppresses bract formation in *Arabidopsis* (Nilsson *et al.*, 1998). Finally, a recessive mutation in the *BRACTEA* (*BRA*) *Arabidopsis* locus results in the retention of bracts and development of flowers subtended by leaves and in an *Antirrhinum*-like inflorescence development (Ezhova, 1999). Therefore, both *Antirrhinum* and *Arabidopsis* form indeterminate monopodial shoots that bear lateral flowers, a growth form that is referred to as a raceme.

The tobacco shoot apical meristem (SAM) converts to a terminal flower upon entry into reproductive development. A few of the youngest axillary meristems also respond to flowering signals, giving rise to sympodially branched flowering shoots, also known as cymes. None of the lateral shoots becomes dominant, resulting in a determinate monopodial habit (figure 5.3C). *Petunia* and tomato initially develop in the same way as tobacco plants, and upon conversion to reproductive development, the primary shoot apical meristem differentiates into a terminal flower. However, the response of the axillary meristems to reproductive signals is different from that observed in tobacco. Only the youngest axillary meristem responds by vigorous growth, displacing the primary shoot apical meristem. The axillary meristem, now at the apical position, develops in a rigidly defined manner to form a sympodial unit. Before differentiating into a floral meristem, the sympodial unit of tomato produces three leaves, while *Petunia* produces only two. These sympodial units are reiterated until the meristem senesces, giving both the tomato and *Petunia* indeterminate growth habits (figure 5.3D and E). Therefore, the sympodial branching in these species can be explained by invoking the following regulatory scheme: termination of the primary meristem growth, i.e. determinancy; differential response of the axillary meristems to 'reproductive signals'; and activation of the rigidly defined developmental programme executed by the axillary meristem, which results in the production of the sympodial unit.

The genetic analysis of *Petunia* shoot architecture has identified several genes required for sympodial growth. Detailed morphological analysis of *Petunia* inflorescence apices suggests that the inflorescence meristem simultaneously generates two bracts before a bifurcation (figure 5.4A; Souer *et al.*, 1998). One half develops as a determinate floral meristem while the other half remains meristematic and initiates two bracts before it bifurcates again. Two mutations have been isolated, *extra petals* (*exp*) and *sympodial* (*sym*), both interrupting bifurcation of the inflorescence meristem (Napoli and Ruehle, 1996; Souer *et al.*, 1998). The resulting inflorescence meristem in these mutant backgrounds differentiates into a floral meristem, terminating further axis development (figure 5.4B). All axillary meristems formed in the axils of leaves terminate with a single flower after a short vegetative development, with none forming a

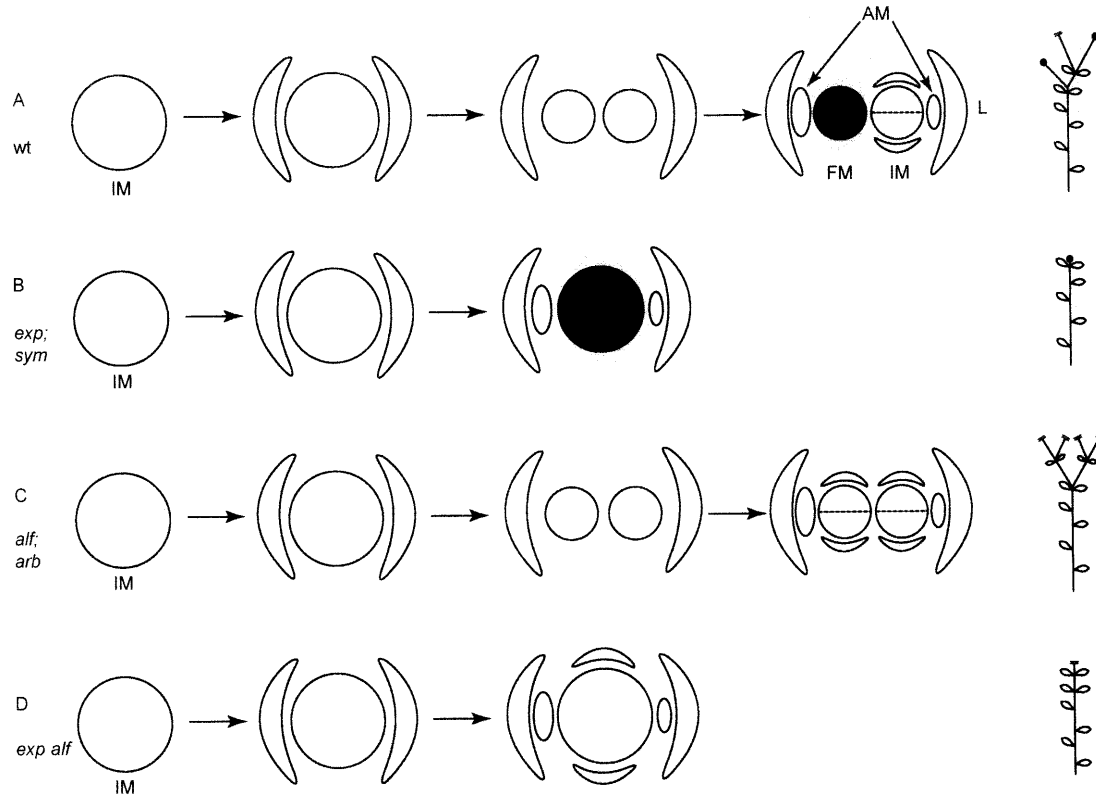


Figure 5.4 Diagrammatic representation of *Petunia* inflorescence meristem development and the resulting shoot form in various genetic backgrounds. A: Wild-type, forms sympodial shoot. B: *exp* and *sym* mutants, form determinate monopodial shoot. C: *alf* and *arb* mutants, form dichotomously branching shoot. D: *exp alf* double mutant, forms indeterminate monopodial shoot. Abbreviations: L, leaf; IM, inflorescence meristem; FM, floral meristem; AM, axillary meristems.

sympodium. As a result, the *exp/sym* mutants form a determinate monopodium, somewhat similar to the branching pattern described for tobacco. The molecular basis of this phenotype is still unknown and awaits the identification of gene products encoded by these loci. Other *Petunia* genes appear to affect meristem identity. *aberrant leaf and flower* (*alf*) is a homolog of *lfy/flo* gene while the *arborescent* (*arb*) has yet to be characterized (Napoli and Ruehle, 1996; Souer *et al.*, 1998). In *alf/arb* mutants, the inflorescent meristem bifurcates giving rise to two meristems, both of which have inflorescence meristem identity. As both meristems continue to develop sympodial units, they give rise to a dichotomously branched axis (figure 5.4C). Double mutant plants which lack both *alf* and *exp* functional gene products have an additive phenotype (figure 5.4D), forming an inflorescence meristem that fails to bifurcate. The resulting meristem is locked in the inflorescence developmental pattern and keeps initiating bract primordia, giving rise to an indeterminate monopodium (Souer *et al.*, 1998). Therefore, changes in expression of only two sets of genes, *exp/sym* and *alf/arb*, can cause a dramatic alteration of the inflorescence architecture in *Petunia*. By analyzing the expression patterns of their homologs in other species, one can assess whether this is the only way of altering inflorescence types.

While the identity of *exp* gene is still unknown, the effect of *lfy/flo*-like genes on inflorescence architecture can be more directly assessed. As in *Petunia*, *lfy*, *flo* and *fa* are required for floral meristem identity in *Arabidopsis*, *Antirrhinum* and tomato (Coen *et al.*, 1990; Weigel *et al.*, 1992; Molinero-Rosales *et al.*, 1999). In *Arabidopsis* and *Antirrhinum*, *lfy* and *flo* do not affect the fate of the primary meristem, and the mutations do not result in a change in the branching type as seen in *Petunia*. Instead, mutations in both *flo* and *lfy* cause more extensive branching as floral meristems convert to axillary inflorescence meristems. Therefore, one can envisage variation in the secondary inflorescence development by altering the timing of activation of *LFY/FLO*-like genes (Coen and Nugent, 1994). Even in tomato, which has sympodial branching, the *fa* mutation has no effect on sympodial development. The *fa* mutation uncouples floral fate from the formation of a cyme by the primary shoot apical meristem and the activation of the youngest axillary meristem to form a sympodium. As with *Petunia*, the primary meristem of *fa* becomes an inflorescence meristem, which continues to form the sympodium. Interestingly, this does not result in the formation of a dichotomously branched axis as seen in *alf/arb* mutants, even though Allen and Sussex (1996) proposed that bifurcation of the primary meristem leads to the formation of sympodial branching in tomato.

One interpretation of the different consequences of *alf/arb* and *fa* on *Petunia* and tomato shoot architectures could be an alteration in the timing of the initiation of the sympodium developmental program in the inflorescence meristem. If the induction of a sympodial fate for the inflorescence meristem occurs prior to the overtopping of the differentiating meristem, then the change

in the meristem fate due to *lfy*-like mutation would result in the formation of two meristems that have the same hierarchy, resulting in a dichotomously branched axis. If, however, sympodial fate is imposed on the inflorescence meristem(s) after one of the meristems has already established its dominance, then the dominant meristem would continue with a sympodial axis and the secondary meristem would develop a sympodial unit in the lateral position, as a side branch, without altering the overall branching scheme. Therefore, *lfy*-like mutant phenotypes are conserved among all the above species. In all cases, the *LFY*-like gene products are required for promoting a floral meristem identity. However, the developmental framework in which floral determination occurs differs between species, so that these mutations produce diverse effects on branching patterns.

The *Arabidopsis* *TERMINAL FLOWER* (*TFL*) and *Antirrhinum* *CENTRORADIALIS* (*CEN*) genes maintain an inflorescence meristem identity by antagonizing the effect of *LFY* and *FLO* gene products. When these genes are inactivated by mutation, an indeterminate growth habit is transformed into a determinate one due to the conversion of the inflorescence meristem to a terminal flower. Thus, the growth habit of *tfl* and *cen* mutant plants resembles that of tobacco. The determinate growth habit of tobacco correlates with the lack of expression of *CEN* homologs (*CET2* and *CET4*) in the SAM. Instead, *CET* genes are localized in the axillary meristems, where their expression coincides with the repression of indeterminate growth (Amaya *et al.*, 1999). In tomato, the *SELF-PRUNING* (*SP*) gene is the ortholog of *CEN* and *TFL1*. The *sp* mutant plants display a determinate shoot phenotype. Determinancy of *sp* mutants is a consequence of the shortening of the length of the sympodial unit. Whilst in wild-type plants the sympodium consists of three leaves before initiation of the inflorescence shoot that terminates the apical meristem, in *sp* mutant plants, the sympodium gradually becomes shorter, until the last sympodium consists of only the inflorescence meristem (Pnueli *et al.*, 1998). Therefore, in all these species, *TFL/CEN*-like genes promote indeterminacy, revealing a conservation of mechanism. As in the case of *LFY*-like genes, the consequence of the alteration of *TFL/CEN*-like gene expression on shoot morphology depends on the species-specific developmental context.

The examples reviewed so far illustrate how simple genetic differences can lead to diverse plant architectures. Though one possible source of these differences between species is the utilization of different gene products to establish the plant form, a more likely explanation is the alteration of the timing and pattern of expression of conserved sets of developmental genes. This latter possibility is exemplified by the *LFY*- and *TFL*-like genes in phylogenetically related species. With the advent of comparative developmental studies, including phylogenetically distant species, it should be possible to assess the functional conservation of these genes in the specification of a plant form and possible alternative ways of specifying the plant architecture.

5.3 Axillary meristem ontogeny

The formation of new axes of growth during postembryonic development by the establishment of axillary meristems is a developmental feature unique to plants. Which cells are capable of organizing axillary meristems? Do they have any unique properties relative to their neighbors? Is their development a continuation of an apical meristem identity or is their fate induced? What is the developmental potential of axillary meristem progenitor cells? Although our understanding of axillary meristem ontogeny is limited, conceptual frameworks underlying axillary meristem specification have been proposed and tools that would allow their testing are becoming increasingly available.

5.3.1 Histological approach to the study of axillary meristem ontogeny

Histological studies on a variety of species have focused on the origin of axillary meristems. According to one interpretation, axillary meristems are derived from a portion of the primary SAM. At the time when each leaf primordium is initiated, the cells on the side of the leaf closer to the meristem (adaxial side) are continuous with the apical meristem. As the leaf primordium increases in size, its cells undergo vacuolation and enlargement, leaving a cluster of meristematic cells called the detached meristem. The detached meristem can therefore be viewed as having developmental continuity with the primary SAM from which it is derived. In some species, it has been proposed that axillary meristems develop from already differentiated tissues (Majumdar 1942; Sharman 1942). The precedent for *de novo* initiation of meristems comes from classic work on shoot regeneration from tissue culture. In particular, it has been established that in many dicotyledonous plants, virtually any differentiated cortical cell of the stem or leaf can give rise to a shoot meristem, if the appropriate ratio of auxin to cytokinin is applied. Because of this totipotent characteristic of plant cells, it is conceivable that an axillary meristem will arise *in planta* whenever and wherever hormone levels cross critical thresholds. This *de novo* formation of axillary meristems therefore invokes postembryonic re-establishment of a meristematic program in the somatic cells of the leaf axil.

The anatomical study of axillary meristem development in Ostrich fern led to the proposition of the detached meristem concept of axillary meristem formation (Wardlaw, 1943). This investigation suggested that the axillary meristem is continuous with the apical meristem, which extends to the youngest leaf primordium (P1) and also downward as ribbons of diminishing width toward the older primordia (P2–P4). In the axil of the fifth leaf, on the stem side, a small group of meristematic cells were observed that were proposed to be part of the region of the apical meristem that originally extended down towards the axil of leaf 5. Other meristematic cells, which at an earlier stage surrounded this residual group, underwent differentiation to separate a small group of

isolated meristematic cells from the apical meristem. These isolated groups of meristematic cells were termed 'detached meristems', although they had no apparent meristematic organization. These cells are indeterminate with respect to their future development, and in the Ostrich fern they can give rise to one or several axillary meristems or can differentiate into parenchymatous tissue. This suggests that these cells have the potential to give rise to the axillary meristem, but that their developmental fate depends on additional signals. Therefore, the detached meristem concept of axillary meristem formation postulates developmental continuity between the primary SAM and axillary meristems, which in conjunction with other signals leads to axillary meristem formation.

Developmental continuity between the primary and axillary meristems has been proposed in a number of flowering plants on the basis of anatomical studies. In some cases, the detached meristem origin of axillary meristems is indisputable. For example, in *Hidrocharis morsus-ranae*, the primary shoot apical meristem initiates the primordium which splits into two parts at the first plastochron: an axillary meristem and leaf domain (Cutter, 1964). In *Hieracium floribundum*, axillary meristem initials are anatomically defined in the leaf primordium axil of the third plastochron, when the leaf primordium is still part of the shoot apical meristem (Yeung and Peterson, 1972). However, in many other species, axillary meristems become anatomically distinguishable in the axils of older leaves that are already separated from the primary shoot apical meristem. In such species, the developmental continuity between axillary meristem progenitor cells and the apical meristem is unclear. Axillary meristem initiation in *Arabidopsis thaliana* exemplifies such a developmental pattern.

In *Arabidopsis*, leaf axil cells are initially indistinguishable from their neighbors and the L1 and L2 cellular layers are continuous between the shoot apical meristem and the youngest leaf primordia (figure 5.5A). Axillary meristem progenitor cells become anatomically defined as a cluster of densely staining small cells in the axils of leaves that have already formed a vascular connection with the main shoot, at approximately the 8th plastochron (Grbic and Bleecker, 2000). When *STM* expression pattern was used to trace the axillary meristem progenitor cells at earlier stages of development, it was found that *STM* mRNA accumulates at lower levels in a subapical domain that includes the axils of the four youngest leaf primordia, and that high levels of *STM* transcripts localize in a few cells of the subhypodermis in the axil of the fifth and sixth leaf primordia marking axillary meristem initials (figure 5.5B) (Grbic and Bleecker, 2000; Long and Barton, 2000). The continuous expression of *STM* mRNA in axillary meristem progenitor cells can be viewed as evidence that, in *Arabidopsis* also, axillary meristems have developmental continuity with the primary SAM, suggesting the detached meristem origin. According to this hypothesis, *STM* expression identifies a field of indeterminate cells below the SAM whose meristematic fate/competence becomes increasingly restricted as tissues develop, until only

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Figure 5.5 Axillary and accessory meristem ontogeny in *Arabidopsis*. A: Longitudinal section of vegetative shoot apex in plant with prolonged vegetative development; the L1 and L2 cellular layers are continuous between the shoot apical meristem (SAM) and the youngest leaf primordia (arrows 1). The area marked with arrows 2 is shown at higher magnification in the inset. Cells in the axil of the older leaf appear smaller relative to surrounding cells. B: Localization of *SHOOTMERISTEMLESS* (*STM*) mRNA at the vegetative shoot apex and the axils of the youngest leaf primordia. *STM* message accumulates at a low level in a broader domain at the apex (arrows 1) and localizes at high levels in a few cells at the axils of older leaves (arrow 2). C: Distribution of *STM* mRNA in the axil of a rosette leaf in which both axillary (ax) and accessory (ac) meristems have formed. D: Scanning electron micrograph of the rosette leaf axil of a *tfl1-2* plant showing axillary (ax) and accessory (ac) buds. E, F and G: Distribution of *STM* mRNA throughout a *tfl1-2* shoot. F and G are close-ups of right and left axil, respectively, of the plant shown in E. Internal scale bars = 100 μ m in A, C and E, 50 μ m in B, F and G, and 200 μ m in D. (Redrawn and adapted from Grbic and Bleecker, 2000. Axillary meristem development in *Arabidopsis thaliana*. *The Plant Journal*, **21** 215-223.)

the axillary meristem progenitor cells retain the capacity to form the meristem proper.

While the experimental data favor the detached meristem model of axillary meristem development in *Arabidopsis*, the important underlying assumption is the functional requirement of the initial *STM* expression for its later accumulation in the axillary meristem progenitor cells. This assumption was tested by analysis of *STM* expression during formation of the accessory meristems that form in the leaf axils away from the apex, and at the time when axillary meristems have already developed (figure 5.5C). Under rare instances, multiple meristems form at the leaf axils in wild-type plants, but they regularly form in the axils of the rosette leaves in the *tfl-1 Arabidopsis* mutant (figure 5.5D). *STM* expression of *tfl-1* shoots, is confined to the floral meristems that develop at the apices of axillary shoots originating in the axils of the youngest rosette leaves (figure 5.5E). In addition, *STM* mRNA accumulates along the length of the elongating stems of the axillary buds in the inner layers of the cortex, reaching the leaf axil where the accessory meristems will form (figure 5.5F). However, the accessory meristems form from cells that are adjacent to those expressing *STM* and do not overlap with them, indicating their *de novo* initiation (figure 5.5G). Therefore, these results do not support the functional requirement for the pre-existing *STM* expression, leaving the axillary meristem ontogeny in *Arabidopsis* unresolved.

However, there is a possibility that both detached meristem and *de novo* initiation modes of axillary meristem formation operate during plant development. It has not been concluded with certainty whether the accessory meristems, which establish in the axils of *tfl1-2* leaves, have the same ontogeny as the primary axillary meristems. Likewise, meristems seldom form in the axils of cotyledons or the first true leaves in wild-type *Arabidopsis* plants (Grbic and Bleecker, 2000), but they regularly arise at these sites when mutations disrupt the normal development of the SAM. For example, plants homozygous for weak alleles of the *STM* locus, *stm-2* and *stm-6*, initiate an embryonic SAM whose development terminates prematurely. These plants subsequently form numerous leaves in the axils of cotyledons (Endrizzi *et al.*, 1996). In addition, *pnh1-2* plants, which form a pin-like structure instead of the primary SAM, often form meristems in the axils of cotyledons (McConnel and Barton, 1995). The origin of these meristems has not been established, but alterations in the apical to basal gradient of auxin, which probably underlies the *de novo* initiation mechanism, might be expected to play a role.

5.3.2 Genetic approaches to the study of axillary meristem ontogeny

The genetic approach to developmental analysis of axillary meristem ontogeny includes the characterization of branching mutants. Lack of axillary meristem development in the branchless mutant class indicates that gene products

impaired in these lines are required for axillary meristem development. In *Arabidopsis*, there are no mutations that specifically alter axillary meristem development. However, there are several mutations that affect its development pleiotropically. For example, plants homozygous for *pinhead 1* (*pnh1-2*), *revoluta 1* (*rev-1*), *auxin-resistant 3* (*axr3*) and *carpel factory* (*caf*) frequently fail to develop axillary buds (McConnel and Barton, 1995; Talbert *et al.*, 1995; Leyser *et al.*, 1996; Jacobsen *et al.*, 1999) leading to a branchless phenotype.

The *lateral suppressor* (*ls*) gene of tomato is more directly involved in axillary meristem development. Mutant *ls* plants do not develop axillary meristems during vegetative (monopodial) growth, but form axillary buds associated with reproductive (sympodial) development (figure 5.6) (Malayer and Guard, 1964). This phenotype is due to a mutation in a graft-non-transmissible gene product, indicating that *Ls* is required in the leaf axils for axillary meristem development (Tucker, 1979). In addition, the morphological defects of the *ls* mutant are accompanied by changes in the levels of several plant hormones. Relative to the wild-type, *ls* plants contain increased levels of gibberellic acid and auxins and decreased levels of cytokinins (Tucker, 1976). The *Ls* gene encodes a new member of the VHIID family of regulatory proteins (Schumacher *et al.*, 1999). The other members of the VHIID family are *SCARECROW* (*SCR*), *GIBBERELLIN INSENSITIVE* (*GAI*) and *REPRESSOR OF GAL-3* (*RGA*), two of which are involved in the repression of gibberellin (GA) signal transduction (Di Laurenzio *et al.*, 1996; Peng *et al.*, 1997; Silverstone *et al.*, 1998).

Assuming the conservation of function between *Ls*, *GAI* and *RGA*, it is conceivable that *Ls* regulates the GA responsiveness in the leaf axils. In this case, the *Ls* gene product may be required to protect the axillary meristem progenitor cells from responding to hormonal signals that induce differentiation of the

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Figure 5.6 Phenotype of leaf axils in: A) wild-type tomato (cv. Moneymaker); and B) *ls* mutant (cv. Antimold B). (Provided by Muller, D. and Theres, K., MPI Fuer Zuechtungsforshung, Koeln, Germany.)

surrounding cells. One way to achieve this is through the localized repression of the GA signal transduction pathway in the leaf axils. However, if *Ls* is required to preserve the axillary meristem progenitor cells in an indeterminate state, how is it possible that axillary meristems form normally during the plant's reproductive development? One proposal compatible with such a scenario is the existence of redundant pathways preventing differentiation of axillary meristem cells. Sympodial growth depends on the extension of the growth axis by axillary buds. Therefore, selection may have favored the redundancy of regulatory pathways that control development of sympodial axillary meristems in order to secure reproductive success. Since branching during vegetative development may not have as profound an effect on reproductive success, its regulation may have remained less complex.

The alternative possibility is that axillary meristem formation is regulated differently during vegetative and reproductive development, and that the *Ls* gene product is required only during the vegetative phase. The timing of axillary meristem initiation and the developmental framework in which axillary meristems form differ between vegetative and reproductive development. It is possible that some of these regulatory networks alter the requirement of axillary meristem cells for the *Ls* gene product. Further characterization of *Ls* normal and ectopic expression, and the requirement for the *Ls* gene product in a background impaired in the GA signaling pathway will allow tests of these hypotheses. Besides understanding the role of *Ls* in axillary meristem formation, the isolation and characterization of additional mutants that specifically affect axillary meristem initiation will be required in order to reconstruct the underlying molecular mechanisms.

5.4 Patterns of axillary meristem development

The architecture of the mature plant is determined by the placement of meristem initials, and the timing of activation of these initials to form higher order shoots. These two processes may be concomitant, as in species in which axillary meristems proceed through an uninterrupted development after their formation, or can be temporarily separated, as in species in which axillary meristem development is initially inhibited upon their formation. In the later case, the axillary meristems may remain dormant throughout the life of the plant, or may be activated at some time during the development of the plant. The mechanisms that regulate these processes can be separated, indicating that the regulatory networks that govern them act independently.

5.4.1 Patterns of axillary meristem initiation

In many species, the pattern of axillary meristem formation depends on the developmental state of the SAM. During reproductive development, axillary

meristems form much closer to the apex than during vegetative growth. This change in the pattern of axillary meristem formation is particularly prominent in species with sympodial branching. For example, in the Rutgers variety of tomato, the sympodium originates from the axillary meristem that forms immediately below the flower primordium, while during vegetative development, axillary meristems become morphologically visible in the axil of the sixth youngest leaf (Malayer and Guard, 1964). Plant species with monopodial branching show a similar pattern of axillary meristem formation. In late-flowering plants of *Arabidopsis*, axillary buds can be observed in the axils of the oldest leaves (figure 5.7A), while there is no sign of their development in the axils of the young leaves. In the axils of the younger leaves (17 to 21) in such plants, the axillary meristems become morphologically apparent as a small raised dome of cells (figure 5.7B). Axillary meristems in the axils of older leaves have already initiated leaf primordia. For example, the axillary meristem in the axil of leaf 22 initiated one primordium, while the axillary meristem in the axil of leaf 25 initiated three. This illustrates the generally observed *acropetal* pattern of development for axillary meristems in a plant with prolonged vegetative development, where the most developed axillary buds are found in the axils of the oldest leaves (Grbic and Bleecker, 2000).

Upon conversion of *Arabidopsis* plants to reproductive development, the pattern of axillary meristem formation changes. Detailed morphological analysis of the initiation patterns at the apex at the transition to reproductive development has revealed that the axillary meristem first initiates in the axil of the youngest leaf primordium, the last leaf primordium initiated by the shoot apical meristem before conversion to reproductive development (figure 5.7C). The axillary meristem at an earlier stage of development can be subsequently seen in the axil of the second youngest leaf primordium (figure 5.7D) and so on, indicating that at the onset of reproductive development, axillary meristems form *basipetally*, in a pattern opposite to that characteristic for the vegetative phase (Hempel and Feldman, 1994).

What is the mechanism that regulates the establishment of these opposing axillary meristem formation patterns? One possibility is that the shoot apex is a source of a repressor of the formation of axillary meristems during the plant's vegetative development. It can be hypothesized that an apically-produced repressor creates a concentration gradient down the shoot, which directly or indirectly inhibits axillary meristem development (figure 5.8A). A good candidate for such a repressor is the plant hormone auxin (Stafstrom, 1993; Cline, 1994). It is conceivable that auxin produced by the SAM forms a gradient of concentrations, declining from tip to base, which below a certain threshold no longer suppresses axillary meristem development. However, the conversion of the SAM from a vegetative to a reproductive state leads to the formation of the opposite, basipetal, gradient of axillary meristem developmental stages. This suggests that in the reproductive state the SAM loses the ability to suppress axillary meristem

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Figure 5.7 Patterns of axillary meristem initiation. A: Vegetative rosette of a late-flowering plant with an axillary bud forming in the axil of a rosette leaf (arrow). B: Transverse section of the vegetative apex depicting an acropetal pattern of axillary meristem developmental stages. Numbers indicate plastochron leaf numbers to assign leaf position relative to the shoot apical meristem (SAM). C and D: Longitudinal sections of the reproductive apex depicting basipetal pattern of axillary meristem developmental stages. Panel C shows an axillary meristem that formed in the axil of the youngest leaf; panel D shows a developing axillary meristem in the axil of the older leaf (arrows). E: SHOOT MERISTEMLESS (STM) mRNA expression in the shoot apex of a plant upon conversion to reproductive development showing axillary meristems formed by the acropetal (arrow 1) and basipetal (arrow 2) initiation gradients. Internal scale bars = 40 μm in C, and 100 μm in D and E. (Redrawn and adapted from Grbic and Bleecker, 2000. Axillary meristem development in *Arabidopsis thaliana*. *The Plant Journal*, **21** 215-223.)

development, or that an identity change at the SAM leads to the establishment of an independent signaling cascade that activates axillary meristem development and overrides the axillary meristem repression. The cytokinins, another plant hormone group with antagonistic effects to auxin, have been implicated in

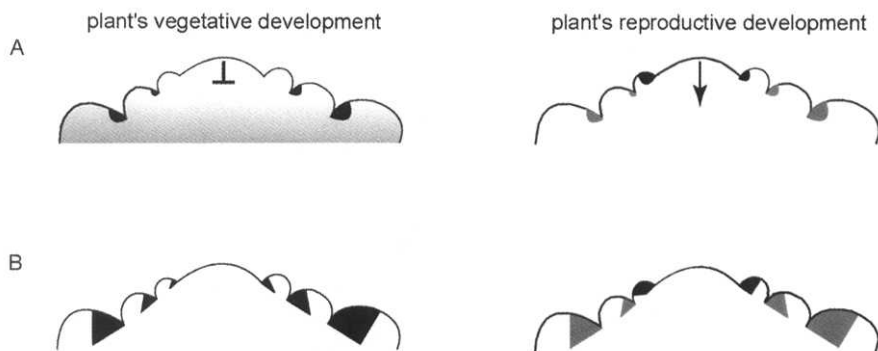


Figure 5.8 Diagrammatic representation of possible cellular events leading to the formation of acropetal and basipetal axillary meristem initiation patterns. A: Axillary meristem formation depends on the apically produced inhibitor of axillary meristem formation during the vegetative plant phase and activator of axillary meristem formation during the reproductive plant phase. B: Patterns of axillary meristem formation reflect differences in the number of axillary meristem progenitor cells between primordia that develop during the plant's vegetative and reproductive development.

axillary meristem development (Stafstrom, 1993; Cline, 1994); however, their role in this developmental process still remains undetermined.

The involvement of auxin in the regulation of the pattern of axillary meristem formation has recently been tested by comparing the timing of axillary meristem formation during vegetative development in the wild-type versus auxin-insensitive *axr1-12* mutant plants (Stirnberg *et al.*, 1999). The *axr1-12* plants have been chosen since the *AXR1* gene is required for many auxin responses, including the repression of axillary meristem development (Lincoln *et al.*, 1990). This analysis revealed that auxin inhibits lateral bud outgrowth, but that the timing of axillary meristem formation is not affected by the loss of the *AXR1* function. These data suggest that a factor other than auxin may exist to create an inhibitory field that suppresses axillary meristem formation close to the vegetative apex.

The establishment of the opposing axillary meristem formation patterns can also be explained by the existence of a different number of axillary meristem competent cells (figure 5.8B). It is possible that vegetative primordia have a leaf bias with only a few axillary meristem progenitor cells in their axils. Thus, only after successive cell divisions do their meristematic domains become anatomically/morphologically apparent. During that time, these primordia are being displaced away from the apex by the newly initiated vegetative primordia, resulting in formation of an axillary meristem in a leaf axil that is well separated from the apex. Upon transition to reproductive development, the lateral primordia initiated by the shoot apical meristem have a meristematic identity. One can hypothesize that signals specifying the meristematic identity affect not

only newly initiated primordia, but also the youngest pre-existing primordia formed at the end of the vegetative phase. The youngest vegetative primordium may be the most responsive to these signals, so that the greatest number of cells gain an axillary meristem identity. The size of the responsive domain would progressively decrease in successively older vegetative primordia as they have already started to develop in a leaf biased developmental pattern. The greatest number of axillary meristem founder cells in the youngest vegetative primordium would require the shortest time to morphologically organize an axillary meristem, while it would take longer for the establishment of axillary meristems in successively older primordia, resulting in a basipetal gradient of developmental stages. The identification of signals that impose meristematic fate on reproductive primordia will allow testing of this hypothesis.

5.4.2 *Patterns of axillary meristem outgrowth*

While the initiation of an axillary meristem is an important prerequisite for branch formation, the patterning of axillary meristem outgrowth is the ultimate determinate of the final plant form. The regulation of the axillary bud outgrowth has traditionally been studied in relationship to the primary shoot apical meristem development, in a process known as apical dominance. This concept has been the subject of many reviews (Cline, 1994, 1997; Stafstrom, 1995; Napolil *et al.*, 1999) and is discussed only briefly here.

The concept of apical dominance postulates that the primary shoot apical meristem controls the outgrowth of lateral buds through the synthesis of a repressor, which moves basipetally down the shoot into lateral buds to inhibit their growth. Consistent with this hypothesis is the bud outgrowth that occurs upon removal of the apex. In addition, experiments by Thimann and Skoog (1934) have indicated that the apically produced repressor may be auxin, since the replacement of the apex with exogenous auxin prevents bud outgrowth. Several other complementary approaches have implicated auxin in the regulation of axillary bud outgrowth. The overexpression of *tryptophan monooxidase* (*iaaM*) and *indoleacetamide hydrolase* (*iaaH*), auxin biosynthetic enzymes from *Agrobacterium tumefaciens* in tobacco, resulted in increased levels of the endogenous auxin and development of plants without branches (Sitbon *et al.*, 1992). Decreases in endogenous auxin levels have been achieved by overexpressing the *indole-3-acetic acid lysine synthetase* (*iaaL*) gene from *Pseudomonas savastanoi* in tobacco plants. In these plants, the endogenously produced auxin was inactivated via conjugation with lysine, which resulted in the release of the apical dominance and the outgrowth of the axillary buds (Romano *et al.*, 1991). Auxin response mutants from *Arabidopsis* confirm the link between auxin and apical dominance. For example, the auxin insensitive mutant, *axr1-12*, has a bushy phenotype (Lincoln *et al.*, 1990), while *axr3*, auxin supersensitive mutant, has a branchless phenotype (Leyser *et al.*, 1996).

A second plant hormone postulated to regulate the axillary bud growth is cytokinin. Alteration of endogenous cytokinin in transgenic plants using the *ipt* gene (encoding the *isopentenyl transferase* which catalyzes a commitment step in cytokinin biosynthesis), resulted in increased levels of endogenous cytokinin and increased branching (Medford *et al.*, 1989; Smigocki and Owens, 1989). The mechanism by which cytokinin might influence bud outgrowth is unknown. Some cytokinin mutants, e.g. the *altered meristem program* (*amp1-1*) (Chaudhury *et al.*, 1993), have a pleiotropic phenotype, so it is unclear whether the branching phenotype arises as a primary consequence of altered cytokinin levels or as a secondary consequence of other developmental alterations.

Characterization of mutants with altered axillary bud growth patterns is an alternative approach to gain insight into the regulatory framework underlying bud development. *Petunia* and pea have provided useful experimental systems for isolation and characterization of such mutants. *Decreased apical dominance* (*Dad*) genes in *Petunia* and *Ramosus* (*rms*) genes in pea have been identified as suppressors of bud outgrowth (Blixt, 1976; Apisitwanich *et al.*, 1992; Arumingtyas *et al.*, 1992; Napoli and Ruehle, 1996; Rameau *et al.*, 1997; Symons and Murfet, 1997). *dad* mutants which comprise three genetic loci: *dad1*, *dad2* and *dad3*, all show extensive development of the first-order branches, while *dad1-1* and *dad2-1* show a second-order branching as well. Grafting experiments indicate that roots or hypocotyls are the source of a diffusible inhibitor of branching whose effect is diminished in the *dad1* mutant (Napoli, 1996). A total of 26 mutants define five *rms* loci. *Rms1* and *Rms2* appear to control the graft-transmissible substances produced in a root and shoot, while *Rms3* and *Rms4* act shoot-autonomously (Beveridge *et al.*, 1994, 1996, 1997). *TEOSINTE BRANCHED 1* (*tb1*) from maize is also implicated in axillary bud development. It encodes a putative transcription factor that represses the growth of axillary buds at lower nodes (Doebley *et al.*, 1997). Therefore, the branching mutants establish the existence of diffusible signals (other than auxin and cytokinin) and axil-autonomous gene products that mediate the response to these signals. However, the components of this pathway are still unknown. Given the complexity of possible interactions, only the characterization of gene cascades, consisting of genes such as *Tb1*, *dad* and *rms*, can provide an insight into the mechanism underlying bud development.

The identification of cDNAs that are specifically expressed in either dormant or growing axillary buds is another approach to understanding genetic circuitry that either regulates or executes these opposing bud developmental states. Pea has been a very useful system for such studies, since axillary meristems usually remain dormant on the intact plant, and their growth can be induced by decapitation of the apex (Stafstrom, 1995). Several transcripts have been identified, whose relative abundance correlates with bud dormancy. For example, the level of *PsDRM1* transcript declines 20-fold as a bud starts growing, but quickly reaccumulates in a bud that converts back to the dormant state (Stafstrom *et al.*,

1998). *PsDRM1* is 75% identical to the auxin-repressed gene from strawberry, further supporting the model in which auxin suppresses bud outgrowth in pea. Another gene, *PsADI*, has similar characteristics. *PsADI* protein is present in dormant buds, and upon decapitation, it disappears acropetally in a pattern that parallels bud activation (Madoka and Mori, 2000). Future characterization of regulatory sequences, and the biological role of dormancy-associated cDNAs, coupled with mutant and candidate gene analyses, will over time lead to the elucidation of the network regulating bud outgrowth.

5.5 Positional determinates of axillary meristems

Axillary meristems form on the adaxial side of the leaf (closer to the shoot apex). Several observations indicate the role of the adaxial cell fate in the promotion of axillary meristem formation. First, mutants with altered leaf polarity display changes in the pattern of axillary meristem formation. Waites and Hudson (1995) described the recessive *phantastica* (*phan*) mutants of *Antirrhinum*, which possess radially symmetric leaves that display an abaxial cell identity around the leaf circumference. This alteration in leaf polarity also correlates with the lack of axillary meristem formation in the most severely affected leaves. The *Arabidopsis* mutant, *phabulosa-1d* (*phb-1d*), has the opposite phenotype. The *phb-1d* mutant plants form radially symmetrical leaves that have adaxial identity around their circumference (McConnel and Barton, 1998). The adaxialized leaves form axillary meristems at the ectopic position around their circumference, reinforcing the linkage between adaxial cell fate and axillary meristem formation (for more detailed discussion see chapter 7 of this volume). In these radially symmetrical leaves, a ring of the *STM* expression forms, marking an early step in the formation of the shoot meristems that surround the leaf base. Therefore, analysis of mutants with altered leaf polarity indicates that adaxial cell fate is both required and sufficient for the axillary meristem formation at the base of the leaf.

In addition, the ectopic expression of *KNOTTED-1* (*KN1*) and *kn-1*-like gene *KNAT1* in tobacco and *Arabidopsis*, respectively, led to the formation of ectopic meristems only on the adaxial side of the leaf blade, despite the constitutive overexpression of the homeobox genes (Sinha *et al.*, 1993; Chuck *et al.*, 1996). It should be noted that these ectopic meristems form over the vasculature, indicating that either the 35S promoter that has been used in these studies has stronger expression near vascular tissue or that vascular development may provide additional factors that limit the position of meristems on the leaves. Vascular strands themselves are polarized within the leaf, with xylem located adaxial to phloem. In the *Antirrhinum phan* mutant, abaxialization of the leaf affects the vascular strands as well, resulting in vascular tissue in which phloem surrounds xylem (Waites and Hudson, 1995). The opposite is true in the *phb-1d*

Arabidopsis mutant, where the xylem surrounds phloem cells (McConnel and Barton, 1998). Therefore, while adaxial cell fate is required for the specification of the axillary meristems, the mechanism by which this polarity determines the site of the meristem formation remains elusive.

Axillary meristems form at the adaxial leaf base at the site closest to vasculature, but is this the only site at which meristems can form on leaves? If alternative sites exist, are these sites specified by the same positional determinants? The formation of inflorescences on leaves, leaves on leaves, vegetative buds on leaves and embryo-like structures on leaves is called 'epiphyllly' and has been described in many angiosperms (reviewed in Dickinson, 1978). The positional relationship between the leaf (that has a normally developing axillary meristem at the leaf base) and epiphyllous structure(s) is shown in figure 5.9. In most cases, an epiphyllous structure arises from a meristematic tissue that forms at the leaf blade. In the majority of cases, epiphyllous meristems form on the adaxial side of the leaf blade in close association with the vasculature, except in a palm, *Chrysalidocarpus*, where the epiphyllous branch forms on the abaxial side of the leaf (Fisher, 1971). Epiphyllous structures can also form on the leaf directly, without the formation of the meristem as an intermediary developmental stage. This is exemplified in *Begonia hispida* var. *cucullifera*, where leaves form directly from leaves, and in *Malaxis paludosa*, in which foliar embryos arrested in an early stage of embryogenesis form at the leaf tip (Taylor, 1967; Maier and Sattler, 1976; Sattler and Maier, 1976). Formation of leaves directly from leaves can be phenocopied in *Arabidopsis* by cultivation of leaf segments in a media supplemented with cytokinin and auxin, and in tobacco plants expressing the *ipt* gene (Estruch *et al.*, 1991; Tsukaya, 1997). These results suggest that alteration in the level of specific hormones may underlie the formation of epiphyllous leaves in *Begonia*.

The formation of the epiphyllous flower in barley, resulting in a *hooded* phenotype, has been studied in greater detail. The presence of a single dominant allele (*K*) causes the formation of the meristematic tissue at the tip of the lemma (a leaf-like structure within the barley spikelet). Upon formation, the

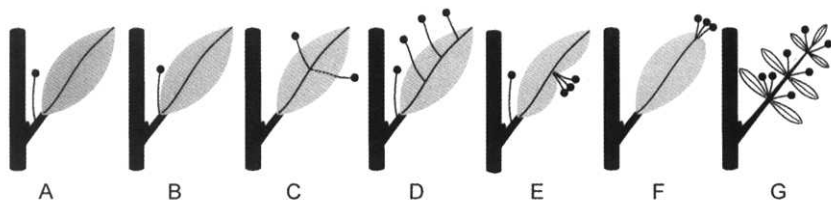


Figure 5.9 Positional relationship between leaf and epiphyllous structure. A: *Turnera* L. (Johnson, 1958); B: *Saururus cernuus* L. (Tucker, 1975); C: *Hordeum vulgare* L. and *Ornithogalum* L. (Stebbins and Yagil, 1966; Samson and Karstens, 1971); D: *Spathicarpa sagittifolia* (Bell, 1991); E: *Polycardia lateralis* (Perrier de la Bathie, 1946); F: *Polycardia phyllanthoides* Lamk. (Perrier de la Bathie, 1946); G: *Lycopersicon esculentum* Mill. (Fukumoto, 1960). (Redrawn from Dickinson, 1978; and Bell, 1991.)

ectopic meristem differentiates into a flower (Stebbins and Yagil, 1966). Taking advantage of the co-linearity between maize and barley chromosomes, Muller *et al.* (1995) cloned the *K* locus and showed that the *hooded* phenotype arises due to the duplication of an intron sequence in a *kn1* barley ortholog *Knox3* homeobox gene. Structural alteration of the *Knox3* gene results in an ectopic expression of *Knox3* at the tip of the lemma, leading to the formation of the ectopic meristem and the epiphyllous flower. Ectopic expression of the maize *kn1* gene in barley phenocopies the *hooded* mutation (Williams-Carrier *et al.*, 1997). In this study, both *35S* and *ubiquitin* promoters were used to express *kn1*. While the *ubiquitin* promoter is active throughout all stages of leaf development, the *KN1* mRNA and protein are only detected in a subset of leaf cells that are able to support meristem formation. These results indicate that *KN1* expression is regulated post-transcriptionally, in a pattern that allows only a subset of cells to express/respond to the *KN1*. Therefore, within the developing leaf, there appear to be restricted domains that can support meristem formation. Mapping domains that are competent to support meristem formation during leaf primordia development may reveal additional factors required for epiphyllous meristems, and thus provide a baseline for further genetic dissection of positional determination of leaf meristem formation.

5.6 Axillary meristem development in plants with no primary shoot apical meristem

Is the primary shoot apical meristem that forms during embryogenesis a prerequisite for the formation of axillary meristems? Could the embryonic shoot apical meristem be regarded as an axillary meristem that forms in the axils of two cotyledons? Can plants develop by reiteration of the units that are formed by axillary meristems? While the answers to these questions are not definite, developmental analysis of *Streptocarpus fanniniae*, *Monophyllaea horsfieldii* and *Wolffia borealis* support the existence of alternative ways to build a plant body that do not require a SAM. In these species, the reiterative units of the plant body are produced by axillary meristems.

In *Streptocarpus fanniniae*, the embryonic shoot meristem forms, but is displaced toward an axillary position of the enlarging cotyledon (figure 5.10A). In this axillary position, this meristem (termed a 'groove meristem') forms detached meristems. Each one of the detached meristems forms a leaf with an axillary groove meristem, which then reiterates the pattern. At the onset of reproductive development, the groove meristem converts into an inflorescence meristem that subdivides into two parts: one gives rise to the leaf with the axillary groove meristem, while the second starts initiating floral meristems (Jong and Burt, 1974). Therefore, the growth pattern of *Streptocarpus fanniniae*, even though initiated from the embryonic shoot apical meristem, exemplifies a morphological novelty that is based on axillary meristems.

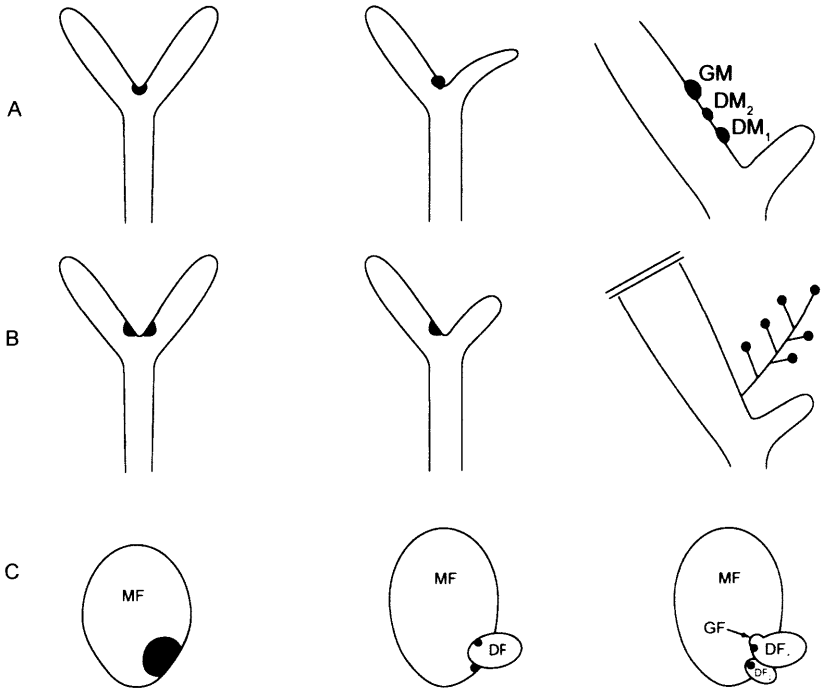


Figure 5.10 Diagrammatic representation of shoot development in: A) *Streptocarpus fanniniiae*; B) *Monophyllaea horsfieldii*; and C) *Wolffia borealis*. Abbreviations: GM, groove meristem; DM, detached meristem; MF, mother frond; DF, daughter frond; GF, granddaughter frond.

A more extreme developmental pattern exists in *Monophyllaea horsfieldii* (figure 5.10B). Embryos of *Monophyllaea* consist of two cotyledons without a shoot apical meristem. However, meristematic cells are present in the axils of cotyledons, as concluded from the anatomical studies and the analysis of the bromodeoxyuridine (BrdU) incorporation (Tsukaya, 1997). Unequal growth of cotyledons results in the enlargement of one cotyledon that retains its axillary meristem, while the development of the second cotyledon, whose meristematic cells differentiate, is arrested. The axillary meristem on the enlarged cotyledon becomes an inflorescence meristem that initiates many flowers. The ontogeny and phenotype of *Monophyllaea* plants resembles the phenotype of the *Arabidopsis* mutant, *pinhead-1* (*pnh1*) (McConnel and Barton, 1995). At the seedling stage, plants homozygous for the *pnh1-2* allele form either a terminal pin-like structure, or none at all, at the position usually occupied by the SAM. In addition, *pnh1-2* plants form adventitious meristems in the axils of cotyledons. These meristems give rise to almost normal looking shoots, except that they frequently fail to develop branches. Further molecular studies, such as

characterization of *PNH* and *STM* orthologs in *Monophylleae*, should provide a better understanding of the *Monophylleae* pattern of development.

The growth pattern of *Wolffia borealis* exemplifies the extreme plant ground plan. It consists of repeating units of leaf (frond) and its axillary meristem (figure 5.10C). Meristematic cells form at the base of the frond of *Wolffia borealis*, which differentiates into a new frond. Daughter fronds develop grand-daughter fronds at their base and so on (Lemon and Posluszny, 2000). Up to four daughter fronds can initiate on a parental frond, resembling the formation of axillary and accessory buds in more conventional leaf axils. Subsequently, these fronds are released from the mother frond, allowing *Wolffia borealis* to reproduce vegetatively. This growth pattern is similar to monochasial cymes (figure 5.10), characteristic of some sympodial inflorescences, except that in *Wolffia borealis* the sympodial meristem differentiates into a leaf whose axillary meristem continues development by forming another leaf with its axillary meristem.

5.7 Concluding remarks

Developmental patterning of axillary meristems is one of the major factors underlying variations in plant morphology. Morphological studies that cover a broad spectrum of phyla have been carried out over the years, establishing a solid foundation for studies aimed at discovering the molecular mechanisms governing the observed variation in plant forms. Identification of the gene products required for axillary meristem formation in model plant organisms, coupled with comparative studies that include species with altered growth patterns should ultimately reveal the mechanism(s) of axillary meristem development and provide insights into the plasticity of the regulatory networks responsible for diverse plant architectures.

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6 Phyllotaxis in higher plants

Didier Reinhardt and Cris Kuhlemeier

6.1 Introduction

In plants, the arrangement of leaves and flowers around the stem is highly regular, resulting in opposite, alternate or spiral arrangements. The pattern of the lateral organs is called phyllotaxis, the Greek word for 'leaf arrangement'. The most widespread phyllotactic arrangements are spiral and distichous (alternate) if one organ is formed per node, or decussate (opposite) if two organs are formed per node. In flowers, the organs are frequently arranged in whorls of 3–5 organs per node. Interestingly, phyllotaxis can change during the course of development of a plant. Usually, such changes involve the transition from decussate to spiral phyllotaxis, where they are often associated with the transition from the vegetative to reproductive phase.

Since the first descriptions of phyllotaxis, the apparent regularity, especially of spiral phyllotaxis, has attracted the attention of scientists in various disciplines. Philosophers and natural scientists were among the first to consider phyllotaxis and to propose models for its regulation. Goethe (1830), for instance, postulated the existence of a general 'spiral tendency in plant vegetation'. Mathematicians have described the regularity of phyllotaxis (Jean, 1994), and developed computer models that can recreate phyllotactic patterns (Meinhardt, 1994; Green, 1996).

It was recognized early that phyllotactic patterns are laid down in the shoot apical meristem, the site of organ formation. Since scientists started to postulate mechanisms for the regulation of phyllotaxis, two main concepts have dominated the field. The first principle holds that the geometry of the apex, and biophysical forces in the meristem determine phyllotaxis (van Iterson, 1907; Schüepf, 1938; Snow and Snow, 1962; Green, 1992, 1996). Alternatively, signal molecules have been proposed to regulate phyllotaxis (Schoute, 1913; Veen and Lindenmayer, 1977; Mitchison 1977; Schwabe, 1984). Recently, the latter view has gained support from studies that analyzed phyllotactic patterning under conditions of perturbed auxin transport (Reinhardt *et al.*, 2000; Vernoux *et al.*, 2000). Based on these and previous studies, we propose that auxin is the trigger of organ formation. Polar auxin transport determines the site of organ formation by regulating auxin distribution in the meristem. A model for the role of primordia in auxin distribution is proposed to account for the reiterative nature of phyllotaxis.

6.2 Phyllotactic patterns in plants

Spiral phyllotaxis is the most common arrangement of leaves in flowering plants and ferns. One leaf is formed per node, with the divergence angle between successive leaves approaching the Fibonacci angle of 137.5° (Steeves and Sussex, 1989; Jean 1994; Lyndon, 1998). Many important model plants in which phyllotaxis has been studied (for example, *Arabidopsis*, tomato, tobacco, lupin, sunflower, poplar) exhibit spiral phyllotaxis. More ancestral plants, such as gymnosperms (for example, *Araucaria*, *Pinus* and *Ginkgo*), as well as ferns (e.g. *Dryopteris* and *Osmunda*) also have spiral phyllotaxis. Given that most of the theories on the regulation of leaf arrangement have been developed in spiral systems, the present review focuses mainly on this form of phyllotaxis.

The sequence in which organs of spiral plants are formed is called the generative spiral. In addition, the arrangement of lateral organs can be described with sets of spirals, called contact parastichies, that run in opposing directions and intersect in the centers of the primordia (figure 6.1) (Steeves and Sussex, 1989; Jean, 1994; Lyndon, 1998). The numbers of spirals that can be observed in each direction in a given spiral system are represented by consecutive numbers in the Fibonacci series, in which each number is the sum of the two preceding numbers, i.e. 1, 1, 2, 3, 5, 8, 13, 21, etc. For example, in the spiral depicted in figure 6.1A, 13 spirals can be drawn from the center in clockwise direction, whereas 21 spirals can be observed in counterclockwise direction, therefore this spiral system exhibits a (13 + 21) phyllotactic pattern.

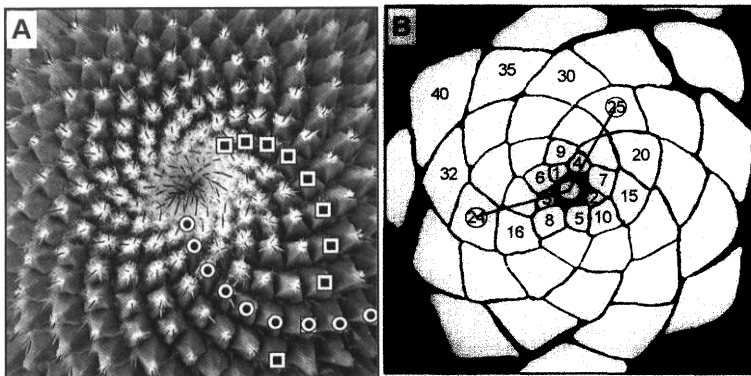


Figure 6.1 Spiral patterns in plants. A: A cactus exhibiting a (13 + 21) spiral pattern. One respective parastichy in each direction is represented by circles and squares. B: Schematic representation of a (5 + 8) spiral pattern. The series of leaf formation is shown with consecutive numbers, with 1 representing the youngest primordium. The divergence angle is the angle between successive primordia (represented by black lines from primordia 24 and 25 to the center). The plastochron ratio is the ratio of the relative distances of consecutive primordia from the center (e.g. distance of 25 divided by distance of 24). (After Church, 1904.)

The principal parameters that determine the phyllotactic pattern are the divergence angle (DA) between successive leaves and the plastochron ratio (PR) (figure 6.1B) (for review, see Richards, 1951; Steeves and Sussex, 1989; Callos and Medford, 1994; Jean, 1994). The divergence angle is defined as the smallest angle between successive leaves (e.g. in figure 6.1B it is 134° between leaves 24 and 25). The PR is defined as the ratio between the distances of successive primordia from the meristem center (e.g. in figure 6.1B, the PR can be given as the distance of leaf 25 from the center divided by the distance of leaf 24 from the center). The PR is therefore a measure of the radial expansion of the shoot apex per plastochron. Since the divergence angle is more or less constant, phyllotactic patterns in spiral plants are mostly determined by the PR. The PR is strongly influenced by the shape and the size of primordia relative to the meristem. If the organs are small relative to their distance from the meristem center, the PR will be close to 1, and a spiral pattern with high numbers of parastichies will be generated (Williams, 1975; Jean, 1994). For example, in the sunflower capitulum which forms small floret primordia, a phyllotactic pattern of up to $(55+89)$ can be observed (Jean, 1994). In contrast, a potato vegetative meristem forms relatively large leaf primordia, leading to a PR of approximately 1.3, and a spiral pattern of $(2+3)$ (Steeves and Sussex, 1989).

Box 6.1 Mathematical models of phyllotaxis

The mathematical analysis of spiral phyllotaxis describes the arrangement and packing of the developing organs around the shoot axis and it emphasizes the regularity and precision of the patterns (Richards, 1951; Erickson and Michelini, 1957; Steeves and Sussex, 1989; Callos and Medford, 1994; Jean, 1994). However, if mathematical models focus on the geometry of the arrangement without considering the underlying cellular and molecular mechanisms, they remain descriptive, and their contribution to the elucidation of phyllotaxis can only be limited. In contrast, mathematical models that envisage chemical or physical principles in the regulation of phyllotaxis (Mitchison, 1977; Veen and Lindenmayer, 1977; Meinhardt, 1994; Green, 1996) are valuable tools, since they make predictions that can be tested experimentally (see sections 6.5 and 6.7).

In distichous or alternate phyllotaxis, one leaf is formed per node with a divergence angle of 180° , resulting in two rows of alternating leaves. Distichous phyllotaxis is characteristic of the grasses, among which the best-studied example is maize (Sharman, 1942, 1947; Veit *et al.*, 1998; Jackson and Hake, 1999). *Pisum sativum* is an example of a dicotyledonous plant with distichous phyllotaxis, in which leaf formation has been studied in detail (Lyndon, 1998).

In whorled phyllotaxis, two or more leaves are formed per node. The simplest case of whorled phyllotaxis is represented by decussate plants. In

decussate or opposite phyllotaxis, whorls of two leaves are formed per node, with each positioned at opposite sides of the stem. The divergence angle between successive leaf pairs is 90° . Decussate phyllotaxis is characteristic of the Lamiaceae (e.g. *Coleus*, *Mentha* and *Salvia*). Other examples of decussate genera are *Antirrhinum*, *Anagallis*, *Hypericum*, *Vinca* and *Acer*. Interestingly, some decussate plants, such as *Salvia*, retain the decussate pattern throughout their entire life span, whereas others such as *Antirrhinum* undergo a transition to spiral phyllotaxis after induction of flowering (see section 6.3).

After decussate, the next highest order whorled system is tricussate (trimerous) phyllotaxis, in which three leaves are formed per node (e.g. *Nerium oleander*). These leaves are positioned symmetrically at 120° from each other, and the divergence angle between whorls of successive nodes is 60° . Up to ten leaves per node are formed by plants of the genus *Galium* (e.g. *Galium verum*). As a general rule, the leaves in whorled systems are formed above the gaps between the leaves of the preceding whorl.

Flowers commonly exhibit whorled phyllotaxis. The standard flower consists of four concentric whorls, the first (outermost) whorl consisting of sepals, the second whorl consisting of petals, the third whorl consisting of stamens, and the fourth (innermost) whorl consisting of carpels (for a review of flower anatomy see Weberling, 1989). Interestingly, the sepals of angiosperm flowers usually have a different ontogeny from the petals. The sepals are formed successively in a spiral sequence, but with little vertical growth between them, therefore resulting (secondarily) in a whorled arrangement. In contrast, the petals of most plants initiate more or less simultaneously, and thus constitute true whorls (Endress, 1994). Normally, the number of organs per whorl is strictly regulated, resulting, for example, in trimerous phyllotaxis in flowers of the Liliaceae, in quadrimerous phyllotaxis in the two outer whorls of crucifer flowers, such as *Arabidopsis*, and pentamerous phyllotaxis in the two outer whorls of flowers in Rosaceae and Solanaceae.

6.3 Changes in phyllotaxis

Although phyllotactic systems are remarkably stable, the phyllotactic patterns can change. Every dicotyledonous plant starts as a bilateral symmetric embryo with a pair of cotyledons. Therefore, the initial phyllotaxis of dicotyledonous plants can be considered to be decussate. Many spiral plants, such as *Arabidopsis*, form a first pair of true leaves more or less simultaneously at opposite sides of the SAM with a divergence angle to the cotyledons of approximately 90° , as in decussate phyllotaxis. *Arabidopsis* seedlings then gradually turn to spiral phyllotaxis (Medford *et al.*, 1992). When spiral phyllotaxis has been established, the plastochron ratio usually decreases during the course of development (Williams, 1975; Erickson and Meicenheimer, 1977). This is mainly due to the increasing

diameter of the apex. The decreasing plastochron ratio, then causes the switch to higher order spiral patterns. For example, in flax, the leaf arrangement changes from a (3+5) to a (5+8) spiral pattern (Williams, 1975).

Decussate plants maintain the initial pattern throughout vegetative growth, and sometimes during their entire life cycle (e.g. Lamiaceae). However, many decussate plants, such as snapdragon (*Antirrhinum majus*) or *Epilobium* change to spiral phyllotaxis during the transition to flowering (Meicenheimer, 1982). Interestingly, the genus *Helianthus* exhibits both, with members that remain decussate during the entire vegetative phase (e.g. *Helianthus microcephalus* and *Helianthus tuberosus*) and only turn to spiral phyllotaxis at the onset of flowering, while other members establish spiral phyllotaxis after formation of a few leaf pairs (e.g. *Helianthus annuus*). Therefore, decussate and spiral phyllotaxis may in some cases share a common basis, and only the timing of transition may distinguish them.

Besides the developmental changes in phyllotaxis, spontaneous changes can occur in nature. Occasionally, decussate plants produce a supernumerary organ per whorl in several successive whorls, resulting in stable tricussate phyllotaxis (figure 6.2). This can involve only a branch (in woody plants, such as *Acer*, *Cornus*, *Ailanthus*) or a whole plant (e.g. *Helianthus tuberosus*). The tendency to form supernumerary organs is also found in flowers of some plant species, for example in tomato, which occasionally forms flowers with six petals instead of five, or in tulips, where flowers with eight (2×4) instead of six (2×3) perigon leaves can be found (Reinhardt and Kuhlemeier, unpublished).

Numerous mutations have been described that lead to changes in phyllotaxis (see section 6.6). Changes in phyllotaxis can also be evoked with chemical growth regulators and by physical interference with the meristem (see section 6.7).

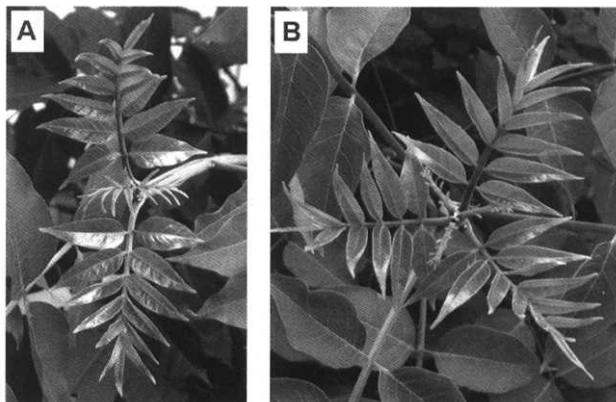


Figure 6.2 Spontaneous switch of phyllotaxis in a decussate plant. A: Decussate branch of an *Ailanthus altissima* tree. B: Tricussate branch of the plant shown in (A).

6.4 Anatomy and function of the shoot apical meristem

Leaves and flowers are initiated at the distal tip of the shoot in a specialized structure, the shoot apical meristem (SAM) (Steeves and Sussex, 1989; Medford, 1992; Lyndon, 1998; see also figure 6.3A). In addition to its function in lateral organ formation, the SAM continuously produces stem tissues and maintains a pool of undifferentiated cells for self-perpetuation. Because the patterns of leaves and flowers that are ultimately revealed on the developing plant are laid down in the meristem, in order to understand the basis of phyllotactic regulation, it is necessary to be familiar with the anatomy and function of the SAM. The anatomy of the meristems of higher plants exhibits some variability (Popham, 1951), but meristems of most plants share several common features that are summarized below. Based on functional, histological and cellular criteria, meristem organization has been described with models of cellular layers and with models of zonation, and these have been described in some detail in chapter 2 of this volume.

6.4.1 *The layers of the meristem*

The meristem of most higher plants exhibits a layered organization (figure 6.3B) (Steeves and Sussex, 1989). The superficial layer, called L_1 , represents the epidermis of the meristem. The cells in L_1 divide anticlinally, that is, the new cross walls are oriented perpendicularly to the surface of the meristem. The L_1 gives rise to the epidermis of the entire shoot, including the stomates and

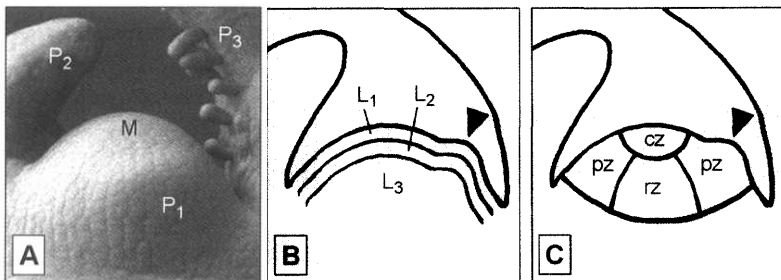


Figure 6.3 Organization of the shoot apical meristem (SAM). A: Tomato apex showing the shoot apical meristem (M) and the three youngest primordia (P1–P3) in clockwise spiral phyllotaxis. The youngest primordium is just being initiated at the flank (towards the viewer), the second youngest is visible on the left. B: Schematic representation of a tomato apex as in (A), showing the layers of the meristem (L_1 , L_2 and L_3). All three layers contribute to the youngest primordium that bulges out at the right side of the flank (arrowhead). C: Schematic representation of a tomato apex as in (A), showing the zonal organization of the meristem. The central zone (cz) is located at the summit of the meristem. The peripheral zone (pz) surrounds the cz, forming a ring-shaped region adjacent to the cz. The rib zone (rz) is located immediately below the cz (and interior to the pz). A primordium is initiated from the right side of the peripheral zone (arrowhead).

trichomes. The cells in the subepidermal layer, L_2 , divide mostly anticlinally like L_1 , however, it has been observed that cells in L_2 divide periclinally early in the process of organ initiation (Lyndon, 1998). The L_2 layer produces the mesophyll, part of the ground tissues, and gives rise to the sporogenic tissues in the flowers. Thus, the products of meiosis are derived from L_2 , that is, the genotype of L_2 cells determines the genotype of the gametes, whereas the genotype of L_1 and L_3 are not transmitted to the next generation. In the L_3 layer, the basal portion of the SAM, cell division has no preferred orientation. The tissues derived from L_3 give rise to the central tissues in stems and in lateral organs. Traditionally, the outer (superficial) cell layers (L_1 and L_2) that divide mostly anticlinally have been called the 'tunica' (the latin name for the roman shirt-like dress), whereas the core of the meristem is referred to as 'corpus' (the latin word for body) (Steeves and Sussex, 1989). For further discussion on meristem organization, refer to chapter 2 of this volume).

6.4.2 *The zones of the meristem*

Leaves are never initiated in the center of the meristem but always on its flank. Therefore, the meristem can be subdivided along its apical-basal axis into a central zone (cz), which remains undifferentiated, and a ring-shaped peripheral zone (pz), in which organ formation occurs (figure 6.3C) (Steeves and Sussex, 1989). Interior to the pz and below the cz, the rib zone (rz) is located; in this region, the central tissues of the stem are formed. The concept of a functional zonation in central and peripheral zone is also reflected by several cytological, histological and molecular properties of the meristematic regions.

6.4.2.1 *Cell division activity and cytological features*

Cell division frequency is usually lower in the center than in the periphery of the meristem (Steeves and Sussex, 1989; Lyndon, 1998; Laufs *et al.*, 1998b). Based on this finding, the concept of a 'méristème d'attente' has been developed (Buvat, 1952; reviewed in Steeves and Sussex, 1989). It assumes that the 'méristème d'attente', which roughly corresponds to the CZ does not give rise to the cells for leaf and stem growth, but that cell divisions in the 'anneau initial' (corresponding to the PZ) are the only source of new cells. The 'méristème d'attente' is pushed upward by the growing stem, and, only in flowers, it becomes activated to form the inner floral organs.

In an opposing view, the CZ is seen as a pool of undifferentiated cells harboring the apical initials. Due to the slow but incessant proliferation of these apical initials, their descendants are continuously displaced to the flank, where they become available for organ formation (Clark, 1997; Meyerowitz, 1997; Laufs *et al.*, 1998c).

The concept of a 'méristème d'attente' has been criticized for two main reasons. Firstly, although cells in the CZ divide less frequently than in the

periphery, mitoses still occur, and it has been estimated that the frequency of cell division is sufficient to replenish the periphery with cell material for organogenesis (Stewart and Dermen, 1970). Secondly, mutant sector analysis has shown that, in rare cases, a single somatic mutation event in the meristem can lead to a sector that comprises the entire shoot circumference (Furner and Pumfrey, 1992). In such an extreme case, the entire affected part of the shoot can be traced back to one single cell in the center of the meristem. Therefore, it is likely that all the mature plant tissues ultimately derive from cells in the meristem center. Although the cells in the CZ have been compared to stem cells in animals (Steeves and Sussex, 1989), there are also prominent functional differences. In animals, stem cells are set apart early in embryogenesis, and in the adult animal, their function is restricted to the replacement of tissues. In contrast, 'stem cells' in plants are involved in growth and morphogenesis during the entire life cycle of the plant.

In many cases, the cells in the meristem center are larger than the ones in the periphery. They are more vacuolated and exhibit various differences in subcellular organization, reflected in a difference in affinity to staining agents (Nougarède, 1967; Steeves and Sussex, 1989). The differences in cellular organization may be associated with different metabolic activities or different cell division activity in the CZ and PZ.

6.4.2.2 Gene expression

Results from *in situ* hybridization studies have revealed that the expression of many genes is not uniform within the meristem. Genes involved in the cell cycle usually exhibit a punctate expression pattern, which corresponds with the distribution of dividing cells (Brandstätter *et al.*, 1994; Fobert *et al.*, 1994, 1996; Segers *et al.*, 1996). Genes for the lipid transfer protein (LTP) are expressed only in the L₁ layer (Fleming *et al.*, 1993; Sessions *et al.*, 1999). Other genes are expressed in a pattern consistent with the zonation model, that is, they are upregulated in the center or at the flank (Fleming *et al.*, 1993; Nishimura *et al.*, 1999). However, gene expression patterns in the meristem can also vary during the course of development (Fleming and Kuhlemeier, 1994).

In recent years, various genes with a role in meristem function have been identified (see also section 6.6). In many cases, the analysis of gene expression has revealed specific expression patterns within the meristem that reflected the zones of the meristem. *SHOOT MERISTEMLESS* (*STM*), which determines meristem identity, is expressed throughout the meristem except for the sites of incipient organ formation (figure 6.4A, G) (Long *et al.*, 1996; Long and Barton, 2000). *CLAVATA3* (*CLV3*) and *CLAVATA1* (*CLV1*), two genes that regulate the meristem size (Fletcher and Meyerowitz, 2000; see also section 6.6), are expressed in subdomains of the meristem center (figure 6.4B, C) (Fletcher *et al.*, 1999). Whereas *CLV3* is expressed mainly in the tunica, *CLV1* is expressed below *CLV3*, mainly in L₃. The *WUSCHEL* (*WUS*) gene which controls meristem

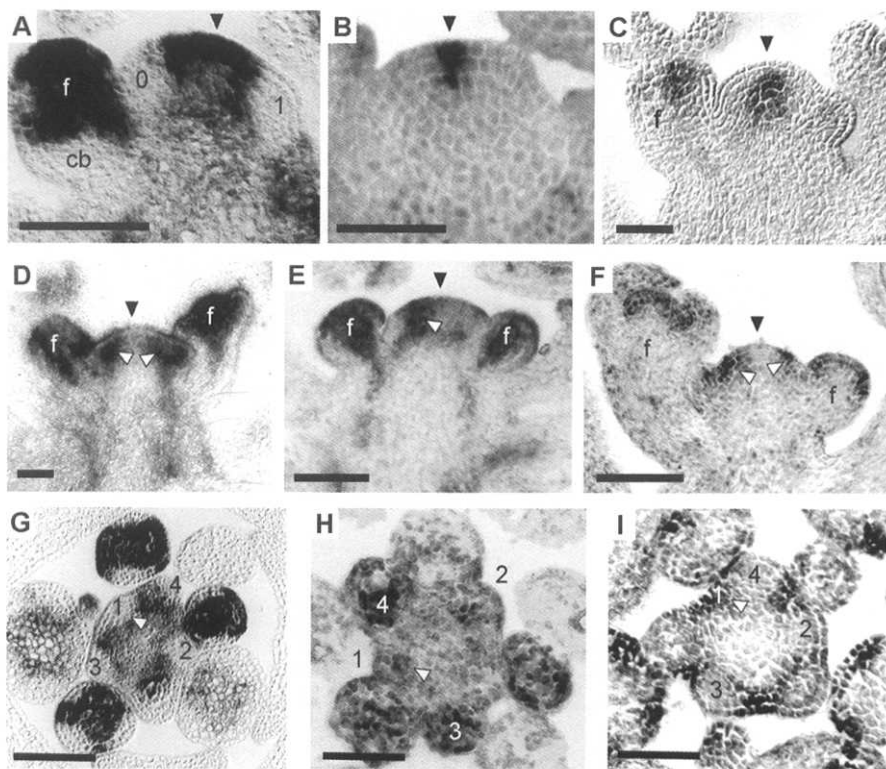


Figure 6.4 Expression patterns of meristem genes in floral apices of *Arabidopsis*. **A:** *SHOOT-MERISTEMLESS (STM)* is expressed throughout the inflorescence (arrowhead) and flower meristem (f), but excluded from young and incipient flower primordia (1 and 0, respectively). In the lower part of the flower primordium (f), *STM* is repressed in a patch that is interpreted as the cryptic bract (cb). (Reproduced with permission from Long and Barton, 2000.) **B:** *CLAVATA3 (CLV3)* is expressed in the meristem centre in L_1 and L_2 , and, to a lesser extent, in L_3 . (Reproduced with permission from Fletcher *et al.*, 1999.) **C:** *CLAVATA1 (CLV1)* is expressed in the meristem centre in L_2 and L_3 , but not in L_1 . In L_3 , the expression domain reaches deeper than *CLV3*. In flower meristems (f), *CLV1* expression is similar to that in the inflorescence meristem. (Reproduced with permission from Christensen *et al.*, 2000.) **D:** *MONOPTEROS (MP)* is expressed in the organogenic region at the flank of the inflorescence meristem (white arrowheads). In developing flowers (f), *MP* is expressed at the site of organogenesis in the inner whorls. (Reproduced with permission from Hardtke and Berleth, 1998.) **E:** *PIN-FORMED1 (PIN1)* is expressed at peripheral sites in the inflorescence meristem (white arrowhead), and in the center of developing flowers (f), in a similar pattern to expression of *MP*. (Reproduced with permission from Vernoux *et al.*, 2000.) **F:** *PINOID (PID)* is expressed at the flank of inflorescence meristems (white arrowheads). In young flower primordia (f), *PID* is also expressed at the flank, and at later stages in developing floral organs. (Reproduced with permission from Christensen *et al.*, 2000.) **G:** *STM* expression reflects phyllotactic pattern in the inflorescence. *STM* is downregulated (arrowhead) at the site of flower initiation (1), and in developing flower primordia (2–4). (Reproduced with permission from Long and Barton, 2000.) **H:** *PIN1* is induced (arrowhead) at the site of organ initiation and in developing flower primordia (2–4). (Reproduced with permission from Vernoux *et al.*, 2000.) **I:** *PID* is induced (arrowhead) at the site of organ initiation. (Reproduced with permission from Christensen *et al.*, 2000.) **A to F:** longitudinal sections; **G to I:** transverse sections; (**A to I:** black arrowheads point to the center of the inflorescence meristem; **A to I:** Internal scale bars = 50 μm .)

maintenance is expressed, like *CLV1*, in the central region of L_3 (Mayer *et al.*, 1998; Schoof *et al.*, 2000). Genes involved in organogenesis, such as *Monopteros*, *Pin-formed1* and *Pinoid* (see also section 6.6), are expressed in the periphery and in young primordia (figure 6.4D–F) (Hardtke and Berleth, 1998; Christensen *et al.*, 2000; Vernoux *et al.*, 2000). Thus, it appears that genes that control meristem perpetuation are expressed in the central zone, whereas genes involved in organogenesis are expressed in the peripheral zone. Interestingly, genes that are expressed in the pz respond to phyllotactic patterning information, for example, *STM* is repressed at the site of organ formation (figure 6.4G; Long *et al.*, 1996, Long and Barton, 2000). In contrast, genes that are required for organ initiation are induced at the site of organ formation (figure 6.4H and I) (Hardtke and Berleth, 1998; Christensen *et al.*, 2000; Vernoux *et al.*, 2000).

6.5 Models for the regulation of phyllotaxis

Organs are always formed at the flank of the meristem at a characteristic distance from the summit, suggesting that some mechanism prevents organogenesis above or below this region. In the radial dimension, organs are formed at characteristic angles from each other, resulting in the phyllotactic arrangements discussed previously. The stunning regularity in phyllotactic arrangements has attracted the attention of many scientists, who have attempted to explain the phenomenon (Church, 1904; van Iterson, 1907; Richards, 1951; Cutter, 1959; Mitchison, 1977; Veen and Lindenmayer, 1977; Jean, 1994; Meinhardt, 1994, 1996; Green, 1996). The principal questions are: 1) How is the site of organ formation selected? 2) How are organs formed? 3) How do pre-existing organs influence the position of new organs? The models for phyllotaxis fall broadly into two categories, namely biophysical and biochemical models, depending on whether the main principle of regulation is envisaged to be of a physical or chemical nature. Although both schools propose different mechanisms of regulation, they both agree with the widely accepted notion that pre-existing primordia influence the sites of future organ formation.

6.5.1 Biophysical models

Various theories of phyllotaxis assume that organogenesis is regulated merely by the geometry of the apex and by tensile and compressive forces that act on the meristem surface. Since these theories attempt to explain phyllotaxis only within the physical parameters of the apex, they are referred to as biophysical models of phyllotaxis. Three of them are discussed here because they have received

particular attention, and are also relevant for the experiments discussed later.

- The ‘theory of the first available space’ states that the timing and positioning of organogenesis is regulated by the availability of a minimal free area on the meristem surface with a minimal distance from the summit and from pre-existing primordia. According to this model, which was first formulated by van Iterson (1907), the geometry of the apex is sufficient to explain phyllotaxis. The simplicity and elegance of this model has stimulated many experiments (Snow and Snow, 1931, 1933, 1962; Steeves and Sussex, 1989; see also section 6.7).
- Two further biophysical theories are based on the idea that differential growth between the tunica layer and the corpus drives organ formation. In the first of these models, originally proposed by Schüepf (1938), the tunica is assumed to grow faster than the corpus. The resulting accumulation of excess tunica surface leads to tangential compression of the tunica, which then responds, passively, with buckling, like a sheet of paper in which the ends are pushed together on a flat plane. The resulting bulge undergoes morphogenesis and develops into an organ. The site of organogenesis would be determined merely by the geometry of the apex. Green has forged this theory into a mathematical model that yields various phyllotactic arrangements found in nature (Green, 1992, 1996; Selker *et al.*, 1992).
- In the second model, growth of the tunica is envisaged to limit meristem growth, leading to compression of the subtending tissues and tangential tension of the tunica. Eventually, the tunica will yield, leading to the formation of a bulge that undergoes morphogenesis (Selker *et al.*, 1992). The site of bulging may be determined either directly by the geometry of the apex, or by local differences in cell wall extensibility or cellulose microfibril orientation (Green, 1985, 1986, 1994; Selker *et al.*, 1992).

6.5.2 Biochemical models

Biochemical models are based on the idea that diffusible signals of chemical nature regulate meristem activity and determine phyllotaxis. The origin of biochemical models can be traced to the ‘field theory’ of Schoute (1913). This model proposes that the primordia, as well as the summit of the meristem, represent centers that are surrounded by fields that may represent gradients of nutrients or signaling molecules.

Mathematical models for a biochemical regulation of phyllotaxis are based on the assumption that competing activities of short-range (autocatalytic) activators and long-range inhibitors determine the sites of local growth and differentiation (Meinhardt, 1994). Depending on the range of activation and inhibition, such models can create various patterns, such as distichous and decussate phyllotaxis

(Meinhardt, 1994), but the mechanism can also be adapted to create spiral phyllotaxis (Meinhardt, 1996).

6.6 Genetics of phyllotaxis

The regularity of phyllotactic patterns makes phyllotaxis an excellent subject for genetic analysis. Mutants affected in organ initiation or with altered organ arrangement can readily be identified in mutant screens. Indeed, mutants affected in most processes of organ formation and patterning have been isolated in mutant screens. Based on the phenotype, they fall broadly into five groups (table 6.1), although many of these are arranged in more than one group. That is, the mutations cause pleiotropic phenotypes. Some of the mutations listed in table 6.1 are discussed below.

6.6.1 *Mutants with defects in meristem initiation or maintenance*

Several mutants, such as *stm*, *wus*, *clv1* and *clv3*, are primarily affected in the establishment or maintenance of the meristem. In general, all mutations that lead to a loss of meristematic cells (e.g. *stm*, *wus*, *phd/zll*), or to an accumulation of meristematic cells (e.g. *clv*, *fas*, *fuf*) also indirectly affect organ initiation and arrangement. Such mutants have been reviewed extensively elsewhere (Barton, 1998; Laufs *et al.*, 1998c; Lenhard and Laux, 1999; Bowman and Eshed, 2000; Fletcher and Meyerowitz, 2000), and are described in more detail in chapter 2 of this volume.

6.6.2 *Mutants with defects in meristem organization*

In this class of mutants, the apical meristem is abnormally organized. For example, the layered organization may be lost or cell shape and size may be abnormal. All mutants with changes in meristem organization also exhibit abnormal phyllotaxis. However, in these cases, it is not clear whether the mutants are affected in the process of organ initiation per se, or whether the alterations in phyllotaxis are indirectly caused by the meristem defect. The *forever young* (*fey*) mutant in *Arabidopsis* may serve as an example of this type of mutation. In *fey* plants, phyllotaxis is highly irregular. Rosette leaves are initiated with divergence angles from 29° up to more than 180°, sometimes resulting in reversal of the generative spiral (Callos *et al.*, 1994). However, the meristems also exhibit histological defects, such as abnormally vacuolated cells, single necrotic cells and loss of the normal layered organization. These defects are evident from the earliest seedling stages, indicating that the meristem develops abnormally in the embryos. In most cases, the meristem aborts before flowering and the plant dies. The *FEY* gene is homologous to reductases, and is expressed throughout the plant, with the notable exception of meristematic tissues (Callos *et al.*, 1994).

Table 6.1 Mutants that are affected in organ formation and phyllotaxis

Mutant	Sp.	Phenotype	References
<i>A Mutants with defects in meristem initiation or maintenance</i>			
knotted1 (kn1)	Zm	loss of indeterminacy	Kerstetter <i>et al.</i> , 1997
no apical meristem (nam)	Ph	no meristem	Souer <i>et al.</i> , 1996
shoot meristemless (stm)	At	no meristem	Long <i>et al.</i> , 1996
cup-shaped cotyledon (cuc1/2)	At	no meristem	Aida <i>et al.</i> , 1997; Aida <i>et al.</i> , 1999
wuschel (wus)	At	meristem arrest	Laux <i>et al.</i> , 1996; Mayer <i>et al.</i> , 1998
pinhead (phd)	At	meristem arrest/ differentiation	McConnell and Barton, 1995; Lynn <i>et al.</i> , 1999
zwille (zll; identical to pinhead)	At	meristem arrest/ differentiation	Moussian <i>et al.</i> , 1998
interfascicular fiberless1 (ifl1)	At	meristem termination	Ratcliff <i>et al.</i> , 2000
revoluta (rev; identical to ifl1)	At	meristem termination	Talbert <i>et al.</i> , 1995
clavata1 (clv1)	At	meristem overproliferation	Clark <i>et al.</i> , 1993; Clark <i>et al.</i> , 1997
clavata3 (clv3)	At	meristem overproliferation	Clark <i>et al.</i> , 1995; Fletcher <i>et al.</i> , 1999
fasciata1 (fas1) and fasciata2 (fas2)	At	meristem overproliferation	Leyser and Furer, 1992
fully fasciated (fuf)	At	meristem overproliferation	Medford <i>et al.</i> , 1992
lanceolate (la)	Le	loss of indeterminacy	Mathan and Jenkins, 1962; Caruso, 1968
lateral suppressor (ls)	Le	no axillary meristems formed	Malayer and Guard, 1964; Schumacher <i>et al.</i> , 1999
defective embryo and meristem (dem)	Le	meristem missing/ defective	Keddie <i>et al.</i> , 1998
<i>B Mutants with defects in meristem organization</i>			
forever young (fey)	At	meristem disorganized	Medford <i>et al.</i> , 1992; Callos <i>et al.</i> , 1994
disrupted (dip)	At	meristem disorganized	Medford <i>et al.</i> , 1992
schizoid (shz)	At	meristem degeneration	Medford <i>et al.</i> , 1992
pasticcino1-3 (pas1-3)	At	meristem disorganized	Faure <i>et al.</i> , 1998
pinhead (phd)	At	meristem arrest/ differentiation	McConnell and Barton, 1995; Lynn <i>et al.</i> , 1999
zwille (zll; identical to pinhead)	At	meristem arrest/ differentiation	Moussian <i>et al.</i> , 1998
mgoun1 (mgo1) and mgoun2 (mgo2)	At	meristem oversized/ fasciation	Laufs <i>et al.</i> , 1998a
enhanced response to ABA (era)	At	increased meristem size	Bonetta <i>et al.</i> , 2000
wiggum (identical to era)	At	increased meristem size	Ziegelhoffer <i>et al.</i> , 2000

Table 6.1 (continued)

Mutant	Sp.	Phenotype	References
<i>C Mutants with defects in organ initiation</i>			
pin-formed1 (pin1)	At	no flowers	Okada <i>et al.</i> , 1991; Gälweiler <i>et al.</i> , 1998
pinoid (pid)	At	no flowers	Bennett <i>et al.</i> , 1995; Christensen <i>et al.</i> , 2000
monopteros (mp)	At	no flowers	Przemeck <i>et al.</i> , 1996; Hardtke and Berleth, 1998
arrested development1 (add1)	At	leaf formation blocked	Picket <i>et al.</i> , 1996
<i>D Mutants with defects in organ separation</i>			
no apical meristem (nam)	Ph	fused cotyledons	Souer <i>et al.</i> , 1996
cup-shaped cotyledon1/2 (cuc1/2)	At	fused cotyledons	Aida <i>et al.</i> , 1997; Aida <i>et al.</i> , 1999
pin-formed1 (pin1)	At	fused cotyledons and leaves	Okada <i>et al.</i> , 1991; Gälweiler <i>et al.</i> , 1998
<i>E Mutants with altered organ number or organ position (phyllotaxis)</i>			
terminal ear (te)	Zm	aberrant phyllotactic angles	Veit <i>et al.</i> , 1998
aberrant phyllotaxy (abphyl)	Zm	2 leaves per node	Jackson and Hake, 1999
perianthia (pan)	At	increased petal number	Running and Meyerowitz, 1996; Chuang <i>et al.</i> , 1999
pinoid (pid)	At	3 cotyledons	Bennet <i>et al.</i> , 1995; Christensen <i>et al.</i> , 2000
enhanced response to ABA (era)	At	increased organ number	Bonetta <i>et al.</i> , 2000
wiggum (identical to era)	At	increased organ number	Ziegelhoffer <i>et al.</i> , 2000
clavata1 (clv1)	At	increased organ number	Clark <i>et al.</i> , 1993; Clark <i>et al.</i> , 1997
clavata3 (clv3)	At	increased organ number	Clark <i>et al.</i> , 1995; Fletcher <i>et al.</i> , 1999
ettin (ett)	At	increased organ number	Sessions <i>et al.</i> , 1997
forever young (fey)	At	aberrant phyllotactic angles	Medford <i>et al.</i> , 1992; Callos <i>et al.</i> , 1994
aintegumenta (ant)	At	less floral organs	Elliott <i>et al.</i> , 1996; Klucher <i>et al.</i> , 1996
tousled (tsl)	At	less floral organs	Roe <i>et al.</i> , 1993
altered meristem program (amp-1)	At	1-4 cotyledons	Chaudhury <i>et al.</i> , 1993
no apical meristem (nam)	Ph	increased petal number	Souer <i>et al.</i> , 1996
leafy (lfy)	At	spiral instead of whorled phyllotaxis	Weigel <i>et al.</i> , 1992
floricaula (flo)	Am	spiral instead of whorled phyllotaxis	Carpenter <i>et al.</i> , 1995
squamosa (sqm)	Am	spiral instead of whorled phyllotaxis	Huijser <i>et al.</i> , 1992; Carpenter <i>et al.</i> , 1995

Abbreviations: Sp., Species; Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Ph, *Petunia hybrida*; Zm, *Zea mays*; ABA, abscissic acid.

From the available knowledge it cannot be deduced what the function of the FEY protein might be, and whether the defect in phyllotaxis is direct or indirect.

6.6.3 *Mutants with defects in organ initiation*

The *pin-formed1* (*pin1*), *pinoid* (*pid*), and *monopteros* (*mp*) mutants of *Arabidopsis* are defective in flower initiation, resulting in the formation of naked pin-like inflorescence stalks (Okada *et al.*, 1991; Bennett *et al.*, 1995; Przemeck *et al.*, 1996). In the inflorescence meristem, the mutation specifically blocks organ initiation but not other meristem functions, since stem growth and meristem self-perpetuation are unaffected. This is in contrast to mutants like *add1* and *add2* (Picket *et al.*, 1996), *shz* (Parsons *et al.*, 2000) or *wus* (Mayer *et al.*, 1998), in which all meristem functions are arrested. The mutated genes in *pin1*, *pid*, and *mp* encode a putative auxin efflux carrier (*PIN1*; Gälweiler *et al.*, 1998), a protein kinase involved in auxin responses (*PID*; Christensen *et al.*, 2000), and an auxin-response transcription factor (*MP*; Hardtke and Berleth, 1998), respectively. Moreover, *pin1* and *mp* have reduced auxin transport capacities in the inflorescence stem (Okada *et al.*, 1991; Przemeck *et al.*, 1996). Thus, the mutant phenotypes and the analysis of the cloned genes implicate auxin as a major player in flower initiation. The inflorescence meristem of *pin1* mutant plants is special for two reasons: firstly, it does not exhibit a deregulated phyllotactic pattern but has no pattern at all; and secondly, the defect in organogenesis is separated from basic meristem function. For these reasons, *pin1* has been very useful for the analysis of flower initiation and meristem patterning (see section 6.8).

6.6.4 *Mutants with defects in organ separation*

The *no apical meristem* (*nam*) mutation in *Petunia* results in the formation of fused cotyledons and the failure to initiate a functional SAM during embryogenesis (Souer *et al.*, 1996). However, occasionally, escape shoots are formed. These develop normally during the vegetative phase, but flowers exhibit various defects in organ number, identity and position, resulting in sterility.

The *NAM* gene is expressed at the outer meristem boundary of the embryonic SAM, and at the boundary between floral organ primordia. From the phenotype and expression pattern of the *NAM* gene, it appears that *NAM* defines the boundary between organs and the SAM, and between incipient organs within the meristem, possibly by negatively regulating growth. In the absence of such boundaries, the embryonic SAM cannot be established, although later in vegetative growth *NAM* appears to be dispensable. Although *NAM* itself may not be the earliest determinant of meristem patterning, it certainly responds to patterning information early in the process of organ initiation.

Double mutants in the redundant genes *Cup-shaped cotyledon1* and 2 (*cuc1* and *cuc2*) in *Arabidopsis* exhibit a dramatic seedling phenotype, in that the

cotyledons are completely fused and the SAM is missing (Aida *et al.*, 1997). The *CUC2* gene, which is homologous to *NAM*, is expressed at the boundary between cotyledon primordia in early embryogenesis, and later marks the boundary between the cotyledons and the SAM, a similar expression pattern to *NAM* (Aida *et al.*, 1999). Therefore, *CUC2* (and probably also *CUC1*) appears to serve a similar function to *NAM* in restricting growth between organs. The role of the *CUC*-like genes is described in more detail in chapter 3 of this volume.

In addition to its inflorescence phenotype, which leads to its name (see section 6.6.3.), *pin1* has a severe vegetative phenotype. Leaves are often oversized, fused or cup-shaped, and sometimes even circular, trumpet-shaped leaves are initiated (Okada *et al.*, 1991; Okada and Shimura, 1994; Reinhardt *et al.*, 2000; Reinhardt and Kuhlemeier, unpublished results). Hence, auxin transport is necessary, not only for organ initiation, but also, directly or indirectly, for determination of organ size and organ boundaries. Interestingly, several genes that are normally expressed in a phyllotactic pattern in the meristem are deregulated in *pin1* mutants (Vernoux *et al.*, 2000). For example, *CUC2*, whose normal expression pattern defines organ boundaries, is expressed in the entire peripheral zone in the meristem of *pin1* mutants, indicating that not only organ initiation but also patterning of the meristem requires PIN1 function.

6.6.5 Mutants with altered organ number or organ position (phyllotaxis)

This group of mutants is particularly heterogeneous, since it reflects the complex nature of factors that influence phyllotaxis. For example, the *fey* mutation (see section 6.6.2), conditions a strong phyllotaxis phenotype. However, this effect may well be indirectly caused by the disorganization of the meristem.

Many mutants with enlarged meristems form supernumerary organs, and examples include *clv1* and *clv3*, and *wiggum/era* (Clark *et al.*, 1993, 1995; Bonetta *et al.*, 2000; Ziegelhoffer *et al.*, 2000). In these cases, it is likely that it is not the patterning mechanism itself that is affected by the mutation, but that the patterning mechanism operates on a larger surface, allowing for more organs to be initiated per whorl. An interesting case is represented by the *abphyl* mutant in maize. In *abphyl* plants, the meristem forms two leaves per node instead of one, resulting in a change from distichous to decussate phyllotaxis (Jackson and Hake, 1999). However, this change was only observed in about 50% of the mutant plants, with the rest having normal phyllotaxis. Although the switch from distichous to decussate appears to be a 'clean' transformation from one phyllotactic pattern to another, it is associated with an increase in meristem size. Significantly, in *abphyl* mutant plants with normal phyllotaxis, meristem size is also normal. Moreover, in decussate *abphyl* plants, the SAM sometimes splits, resulting in twin shoots that now exhibit normal distichous phyllotaxis, possibly because after the split, meristem size is decreased (Kerstetter and Hake, 1997). These results indicate that increased meristem

size is at least associated with, if not causal for, altered phyllotaxis in *abphyl* mutants.

Several homeotic mutations in flower meristem identity genes have been reported to cause changes in phyllotaxis (Coen *et al.*, 1990; Huijser *et al.*, 1992; Weigel *et al.*, 1992). For example, in the *leafy* (*lfy*) mutant of *Arabidopsis*, inflorescence shoots are formed in place of flowers. Together with inflorescence identity, these meristems exhibit a spiral instead of the whorled phyllotaxis of normal flowers (Weigel *et al.*, 1992). Similarly, in the *floricaula* (*flo*) and *squamosa* (*squa*) mutants of *Antirrhinum*, flowers are converted into inflorescence shoots that exhibit spiral phyllotaxis (Coen *et al.*, 1990; Huijser *et al.*, 1992; Carpenter *et al.*, 1995). In all these cases, not only phyllotaxis but also determinacy and meristem identity are changed as a consequence of the homeotic transformation. In fact, these meristems exhibit the appropriate phyllotaxis for the new identity. Therefore, the change in phyllotaxis is likely to be the indirect consequence of altered meristem identity due to the homeotic transformation, and not caused by direct interference with the patterning mechanism.

There are at least two mutants, *terminal ear* and *perianthia*, which exhibit altered phyllotaxis without other effects on meristem size or organization. Thus, these mutants may be affected directly in the patterning mechanism that determines phyllotaxis. The *terminal ear* (*te1*) mutation in maize causes several deviations in the development of internodes and leaves (Veit *et al.*, 1998). Leaf number is increased and the internodes are dramatically reduced, resulting in the ear-like appearance of the terminal tassel. In addition, the radial position of leaves deviates from the normal 180°. The *TE1* gene, which encodes a putative RNA-binding protein, is expressed in the apex in semicircular bands marking the tissues between successive leaves in the two rows of leaves. Considering the phenotype with excess leaves, and the fact that leaves in wild-type plants are initiated at the site of lowest *TE1* expression, *TE1* has been proposed to function by inhibiting leaf formation. Consistent with this model, leaves of *te1* mutants appear to initiate higher on the apical dome than those of wild-type plants (Veit, B.E., personal communication).

Plants of *Arabidopsis* with a mutation in the *PERIANTHIA* (*PAN*) gene form pentamerous flowers instead of the tetramerous flowers characteristic of crucifers (Running and Meyerowitz, 1996). Among the patterning mutants, *pan* is significant for several reasons: firstly, *pan* flowers have virtually no defect in organ formation and organ differentiation, and they are fully fertile, that is, the genetic lesion specifically affects the patterning mechanism, not general development; secondly, *pan* floral meristems exhibit normal size and structure, indicating that the change in organ number is not the indirect consequence of altered meristem organization; and thirdly, the mutation results in the conversion to a floral pattern which is commonly found in nature, for example in ancestral plants of the Capparaceae family (Chuang *et al.*, 1999). Therefore, the *PAN* gene may have played a role in the evolution of crucifer flower pattern by reducing

organ number from five to four. The *PAN* gene encodes a putative transcription factor of the bZIP family, however, the function of the PAN protein is still elusive (Chuang *et al.*, 1999).

Box 6.2 Do specific phyllotaxis genes exist?

This survey of the meristem and phyllotaxis mutants shows that most mutants exhibit highly pleiotropic phenotypes. Notably, specific transitions from one phyllotactic pattern to another (e.g. spiral to decussate) are never observed. In many cases, effects on phyllotaxis (organ number or position) are associated with altered meristem size or organization, and thus such effects may well be indirect. The pleiotropic phenotypes of many phyllotaxis mutants, and the rare occurrence of specific patterning mutants, may be due to one or more of the following: 1) mutations in specific patterning genes may be lethal; 2) phyllotaxis may be regulated by genes with redundant functions; 3) the mechanisms that regulate phyllotaxis may be intimately linked with other (unrelated) functions, leading to pleiotropic phenotypes in the mutants; and 4) genes might determine phyllotactic patterns not directly, but indirectly by controlling the parameters of the system that generates the patterns (Douady and Couder, 1996). Thus, phyllotaxis may be a complicated multigenic trait.

In conclusion, genetics is providing an increasingly detailed picture of the regulation of meristem establishment and maintenance (Barton, 1998; Laufs *et al.*, 1998c; Lenhard and Laux, 1999; Bowman and Eshed, 2000; Fletcher and Meyerowitz, 2000; refer also to chapter 2 of this volume). However, genetic studies have not provided a coherent picture of phyllotactic regulation, including an answers to the following principal questions: (i) What mechanism determines the site of organ formation? (ii) How is organ initiation regulated at the cellular level (cell division, cell expansion)? (iii) Which regulatory molecules (growth factors, hormones, morphogens, etc.) are involved in the regulation of phyllotaxis? The analysis of several mutants (*pin1*, *pid*, *mp*, *ett*) has hinted at a central role of auxin in phyllotaxis. However, mutant analysis has not revealed how auxin is involved and what role the corresponding genes may play in organ initiation and spatial patterning.

6.7 Experimental evidence for models of phyllotaxis

Proponents of both biophysical and biochemical models of phyllotaxis have employed mathematical modelling to support their notions (Richards, 1951; Williams, 1975; Mitchison, 1977; Veen and Lindenmayer, 1977; Schwabe, 1984; Meinhardt, 1994; Green, 1996). However, it has been more difficult to obtain direct experimental evidence for the basis of phyllotactic patterning

mechanisms, mainly due to the minute size and the delicacy of the meristem, which is only approximately 100–200 μm in diameter. Nevertheless, microsurgery and pharmacological approaches have been successfully used to gain insight into meristem function.

The most frequently applied experimental approach has been microsurgery. Meristems have been dissected and isolated, and incisions have been made to isolate primordia or parts of the meristem from the remaining meristem (reviewed in Steeves and Sussex, 1989; Callos and Medford, 1994). The rationale for such experiments was to test to what extent meristem development is dependent on the subtending tissues (Ball, 1948; Smith and Murashige, 1970), and how the pre-existing primordia affect subsequent organ formation and the patterning of the apex (Snow and Snow, 1931, 1933, 1962; Wardlaw, 1949).

Ball (1948) isolated the meristem from primordia and vasculature by four deep vertical incisions around the meristem. The meristems that remained on a plug of central pith tissue continued to develop almost normally, although phyllotactic patterning was not analyzed in detail. It was concluded that the meristem is an independent pattern-generating unit that relies on the more mature apical tissues only for basic nutrients. In contrast, complete isolation of meristems disrupted organ formation completely (Smith and Murashige, 1970). Only after about 10 days of culture on medium, such meristems regained the capacity to form leaves, and later, normal spiral phyllotaxis was re-established. Thus, the meristem seems not to be autonomous from the more mature tissues. Although the meristems of Ball (1948) appeared to contain only the apical non-patterned dome, we now know that even in the absence of any morphological or histological signs, the meristem contains a pre-pattern. This is shown by specific gene expression patterns in the meristem (see section 6.4.2.2). Also, it has been concluded, based both on theoretical considerations and on experimentation, that the determination of leaf position is influenced by pre-existing primordia (Steeves and Sussex, 1989). Clearly, regular patterns can only be established if new leaves are positioned relative to pre-existing leaves. Therefore, some form of positional information needs to be provided to the meristem from the primordia.

Experiments analogous to Balls have been carried out with 1-naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport (Reinhardt *et al.*, 2000; see also section 6.8). NPA suppresses leaf formation, whereas meristem self-perpetuation and stem growth are not affected. Apices that were cultured on NPA-containing medium for prolonged times consisted only of a stem with the meristem at the tip, thus lacking any pre-pattern conferred by pre-existing primordia. When such meristems were transferred to medium without NPA, leaf formation was reinitiated but phyllotaxis was abnormal and spiral phyllotaxis was re-established only gradually (Reinhardt *et al.*, 2000). Thus, although pre-existing leaves are not necessary to form new primordia, they do provide spatial information to determine the position of new primordia.

Snow and Snow (1931, 1933) surgically isolated primordia or the site of incipient primordium formation (I_1) from adjacent tissue by incisions in order to understand the role of these tissues in further organ positioning. For instance, if I_1 was isolated, I_2 was initiated at the expected position, however, I_3 was formed closer to I_1 than normal (figure 6.5). Snow and Snow interpreted their findings as evidence for a 'first available space' mechanism (Snow and Snow, 1931, 1933, 1962). They argued that, since I_1 was prevented from occupying its 'normal space' on the meristem, more space was available for I_3 which is initiated in proximity to I_1 . Therefore, I_3 'moved' towards the resulting gap, and was initiated closer to I_1 and almost opposite to I_2 .

However, the results could also be interpreted as evidence for negative signaling from pre-existing primordia. This points to the fact that first-available-space and negative-field models are difficult to distinguish experimentally because they make similar predictions. In both models, the probability of leaf formation increases with the distance from pre-existing primordia.

Wardlaw (1949) performed similar surgical experiments on apices of the fern, *Dryopteris*. Although, in these experiments, the effect of surgical treatments on the site of primordium initiation was similar to the results of the experiments performed by Snow and Snow, the author came to different conclusions. Firstly, the availability of free space seemed not to be limiting, since the primordia of

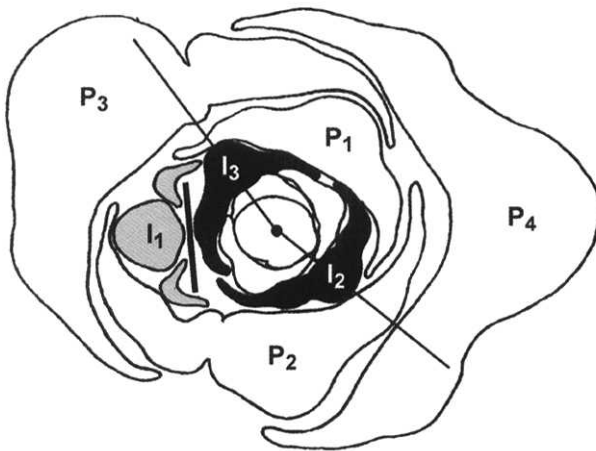


Figure 6.5 Isolation of an incipient leaf primordium (I_1) leads to the displacement of the second next primordium (I_3). In a lupin apex, the site of incipient leaf formation (I_1) was isolated from the rest of the meristem by a vertical incision (represented by a black bar). After 3 weeks, the apex was fixed and sectioned. In addition to I_1 , which grew out after isolation, five new primordia were formed (interior to the black bar). Whereas I_2 is at the expected site, I_3 is closer to I_1 , and almost opposite to I_2 . The divergence angle between I_2 and I_3 is approximately 165° , whereas normally the divergence angle is approximately 136° . (After Snow and Snow, 1931.)

Dryopteris are widely spaced. Secondly, aberrant organ positioning induced by surgical manipulation did not always occur at the site of 'first available space'. Therefore, Wardlaw concluded that signals emanating from primordia were the basis of organ positioning rather than the availability of free space.

One general pitfall of surgical experiments has to be taken into account when interpreting such results. That is, isolating very young primordia from meristems creates wounds, particularly as isolation of the site of incipient leaf formation requires tangential incisions into the meristem. However, the meristem is likely to respond to such insults with wound development and alterations in growth dynamics (Pilkington, 1929; Steeves and Sussex, 1989), effects that would be expected to alter meristem function and phyllotaxis, making conclusions difficult.

Schwabe (1971) used a pharmacological approach to interfere with phyllotaxis. He treated *Chrysanthemum* plants with the auxin transport inhibitor, TIBA. This treatment resulted in a stable transformation from spiral to distichous phyllotaxis. However, not only leaf position, but the whole geometry of the apex was altered. In particular, the vertical distance of leaf primordia was increased. Based on these experiments, Schwabe favored a 'field model' of phyllotaxis, according to which organ positioning is regulated by negative influences from the two youngest primordia (figure 6.6). The author assumed that under natural

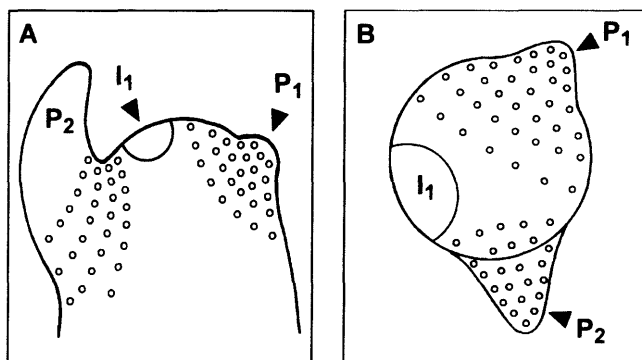


Figure 6.6 A model for phyllotaxis based on inhibitory fields emanating from leaf primordia. A: Shoot apex in a longitudinal representation with two preformed primordia (P_1 and P_2). The primordia produce an inhibitor of leaf formation that diffuses into the meristem (inhibitor represented by small circles). At a certain distance from the primordia, the concentration of inhibitor is low enough to allow leaf formation (I_1). P_2 is lower than P_1 and more remote from the meristem flank, thus the inhibitory influence is weaker than that from P_1 . Consequently, the next primordium will be formed closer to P_2 than P_1 . (After Schwabe, 1971, 1984.) B: The situation is similar to that in (A) but represented as a transverse section at the level of P_1 . Due to its proximity to the meristem, the gradient emanating from P_1 covers a larger surface than the gradient from P_2 , which is lower (indicated by a line between P_2 and the meristem). Therefore, I_1 is closer to P_2 than P_1 , leading to a divergence angle of approximately 137° , characteristic of spiral phyllotaxis.

conditions, the youngest primordium (P_1) had a stronger 'repellent' effect than the second youngest (P_2), so that the incipient primordium (I_1) would be placed closer to P_2 than P_1 (as observed in spiral phyllotaxis). Since TIBA caused an increase in the vertical distance between primordia, he concluded that P_2 became too remote from the meristem to have any inhibitory effect. Consequently, I_1 would be placed as far as possible from P_1 , and after a few plastochrons, stable distichous phyllotaxis would be established. Thus, the effect of TIBA on phyllotaxis was thought to be indirect.

Meichenheimer (1981) studied the effect of inhibitors of auxin transport and action on the decussate (bijugate) plant, *Epilobium*. In these experiments, decussate phyllotaxis was transformed into spiral phyllotaxis by local application of inhibitors to young primordia. Although Meichenheimer concluded, in agreement with Schwabe, that gradients of inhibitors emanating from primordia determine phyllotaxis, the role of auxin was interpreted differently. Whereas Schwabe suggested that auxin played only an indirect role by regulating growth parameters in the apex and by affecting the range of inhibitors in the meristem, Meichenheimer proposed that auxin itself could be the inhibitor.

In addition to auxin transport inhibitors, auxin itself has been applied to shoot apices (Snow and Snow, 1937). In general, application of exogenous auxin has been shown to induce increased primordium size and fusions of primordia (see following section).

6.8 Auxin regulates initiation and radial position of leaves and flowers

Hormones, especially auxin, gibberellin and cytokinin, have long been thought to play a role in meristem development and organ formation (Snow and Snow, 1937; Bedesem, 1958; Kiermayer, 1959, 1960; Schwabe, 1971; Maksymowych and Erickson, 1977; Meichenheimer, 1981; Marc and Hackett, 1992; Chaudhury *et al.*, 1993; Dewitte *et al.*, 1999). However, the specific role of hormones in phyllotaxis has not been established. In many of the earlier studies, the experimental treatments were not sufficiently restricted in time and space, or the time between treatment and sampling was rather long (e.g. a week or more). Therefore, it has been difficult to separate direct and indirect effects of the growth regulators.

Recently, the role of auxin in organ initiation and phyllotaxis was studied using both the auxin transport mutant, *pin-formed1*, and specific inhibitors of polar auxin transport (Reinhardt *et al.*, 2000). In this study, it was shown that the auxin transport inhibitor, NPA, specifically inhibited leaf initiation in tomato meristems. However, meristem perpetuation and stem growth were not affected, since the apices continued to grow, forming pin-like shoots (further referred to as NPA pins). Thus, auxin transport is specifically required for

leaf initiation, but not for general meristem growth. The separation of organ formation from general meristem growth makes it possible to analyze these processes independently. To establish the role of auxin in leaf formation, small droplets of lanolin containing indole-3-acetic acid (IAA) were administered to the flank of NPA pins, at a distance from the summit that corresponds to the distance in natural leaf formation. This treatment induced leaf formation after one day (figure 6.7A; Reinhardt *et al.*, 2000).

Mutations in the *Arabidopsis pin-formed1* (*pin-1*) gene lead to a defect in organ formation in the inflorescence, resulting in pin-shaped stalks resembling tomato apices cultured on auxin transport inhibitors (Okada *et al.*, 1991; see also section 6.6.3). This mutation was recently traced to a putative auxin efflux carrier (Gälweiler *et al.*, 1998), indicating that, as with leaf formation in tomato, flower initiation in the *Arabidopsis* inflorescence requires polar auxin transport. Administration of droplets containing IAA to the flank of *pin-1* meristems induced flower primordia (figure 6.7B; Reinhardt *et al.*, 2000). In both tomato and *Arabidopsis* pins, organs were only induced at the flank of the meristems, as with untreated meristems, but never on the summit or below the flank. Even if IAA was applied to the summit of the meristem, organ formation could not

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Figure 6.7 Auxin induces organ formation on tomato 1-naphthylphthalamic acid (NPA) pins and *Arabidopsis pin-formed1* apices. A: Tomato apices were cultured on medium containing the auxin transport inhibitor NPA. This resulted in the inhibition of leaf formation, whereas stem growth continued, resulting in the formation of a naked pin-like shoot. Local administration of lanolin paste containing 1 mM indole-3-acetic acid (IAA) to the flank of such NPA pins induced leaf primordia at the site of treatment. (Reproduced with permission from Reinhardt *et al.*, 2000.) B: The inflorescence meristem of *Arabidopsis pin1* mutant plants is inhibited in flower formation, resulting in the formation of pin-shaped stalks. Local administration of lanolin paste containing 1 mM IAA to the flank of such inflorescence meristems induced flowers at the site of treatment. (Reproduced with permission from Reinhardt *et al.*, 2000.)

be induced at this position, but only at the flank, indicating that auxin was able to diffuse over this short distance in the absence of polar transport. In the radial dimension, organogenesis always coincided strictly with the site of auxin application. From these observations, a number of conclusions can be drawn:

- Auxin is necessary and sufficient to induce organs at the SAM.
- Since auxin only induced organs at the flank but not in the meristem center, an auxin independent pre-pattern appears to be maintained in pin-meristems functionally equivalent to the zonation observed in untreated meristems, comprising a central zone of undifferentiated stem cells and a peripheral zone that is capable of organogenesis if supplied with auxin. Within the ring-shaped tissue at the flank, auxin could induce organs at any position.
- Auxin was able to induce both leaves on vegetative tomato pins, and flowers on *pin1-1* inflorescence pins. Therefore, auxin appears to be a universal inducer of organogenesis in plants, and other factors in the meristem appear to determine organ identity.

In this context, it is interesting to note that although flowers are formed as lateral structures in phyllotactic patterns like leaves, they are not organs in the strict sense, but they represent determinate lateral meristems (Coen and Nugent, 1994). The results discussed above show that the initiation of leaves and flowers share a common mechanism involving auxin, and therefore they may not represent fundamentally different processes. In most angiosperm families, flowers initiate in the axils of bracts (Coen and Nugent, 1994). In crucifers, such as *Arabidopsis*, however, flowers are not subtended by bracts. It has been proposed that this is a derived condition, and that remnants of cryptic bracts may still be involved in flower formation (Coen and Nugent, 1994; Long and Barton, 2000). Interestingly, in the *leafy* mutant of *Arabidopsis* in which flowers exhibit inflorescence shoot characteristics, the abnormal flowers are occasionally subtended by bracts (Weigel *et al.*, 1992). Therefore, flower formation in *Arabidopsis* may have evolved from a process that is homologous to lateral shoot formation, and in *leafy* mutants, this ancestral pathway of flower formation (including bracts) is revealed by the partial loss of flower identity (Weigel *et al.*, 1992; Coen and Nugent, 1994). According to this idea, it is possible that in the *Arabidopsis* inflorescence meristem, auxin induces primordia that consist of both an abaxial portion that corresponds to a cryptic bract (that is inhibited to grow by the *LEAFY* gene), and an adaxial portion that represents the actual flower primordium. Indeed, the *STM* gene, a marker for meristem tissues, is downregulated on the abaxial side of flower primordia, in a similar way to leaf initiation (Long *et al.*, 1996; Long and Barton, 2000; see also section 6.4.2.2). This region has therefore been interpreted as the cryptic bract. In this context, it would be interesting to see whether auxin could induce leaves on *pin1/leafy* double mutant meristems.

Polar auxin transport is required for leaf and flower initiation. But where is auxin produced, and where is it transported to in order to promote organ initiation? Auxin is thought to be produced in young developing tissues of the shoot, particularly in young leaves (Davies, 1995). However, it is not clear whether auxin is produced in the meristem proper. Therefore, inhibition of auxin transport could lead to either accumulation or depletion of auxin in the meristem, depending on whether or not the meristem is a source of auxin. The fact that exogenous auxin can restore organ formation in pin meristems suggests that these meristems experience auxin depletion rather than accumulation, and that auxin depletion is the reason for inhibited organogenesis. If the inhibition of polar auxin transport leads to depletion of auxin in the SAM, we have to assume that, under normal conditions, acropetal polar auxin transport from subtending tissues regulates organ formation in the meristem. Alternatively, polar auxin transport could lead to reallocation of auxin within the meristem to build up gradients that determine phyllotaxis.

Based on the conclusions above, we proposed that dynamic gradients of auxin in the meristem determine the site of organ formation, and that pre-existing leaves influence these auxin gradients by modulating acropetal auxin transport or auxin distribution within the meristem. If gradients of auxin determine phyllotaxis, the pattern of organ formation would be expected to be sensitive to

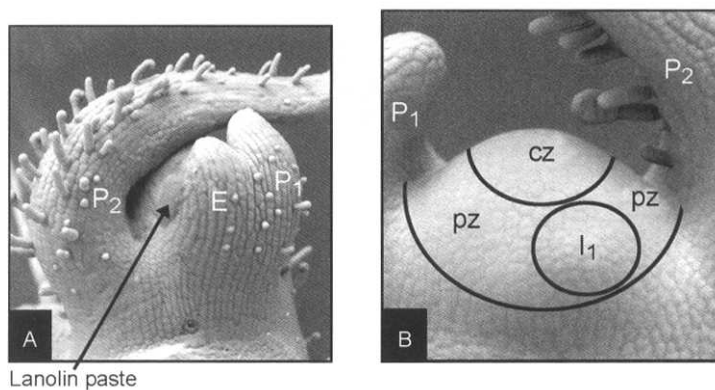


Figure 6.8 Regulation of phyllotaxis by auxin. A: A tomato apex with two preformed leaf primordia (P₁ and P₂) was treated with lanolin paste containing 10 mM indole-3-acetic acid (IAA). The site of application was at I₂, i.e. the site where the second next primordium would normally be formed (between P₁ and P₂). The next primordium (I₁) was expected to form in the back (not visible). Instead, an ectopic primordium was induced at the site of IAA application (E). (Reproduced with permission from Reinhardt *et al.*, 2000.) B: A model for the role of auxin in organ formation. A vegetative apex with two leaf primordia (P₁ and P₂) and the meristem, consisting of the peripheral zone pz and the central zone cz. This apical-basal prepattern is auxin-independent. Auxin accumulation at the site of incipient leaf formation (I₁) induces the cells in the peripheral zone to form a leaf. The pre-existing leaf primordia (P₁ and P₂) determine the site of auxin accumulation.

exogenous application of auxin. This is indeed the case, since exogenous auxin is able to induce ectopic primordia on the flank of otherwise untreated meristems (figure 6.8A; Reinhardt *et al.*, 2000). This indicates that in normal meristems, as in tomato and *Arabidopsis* pins, various positions around the meristem flank are competent of organogenesis if supplied with auxin. This suggests that it is the availability of auxin that determines organ position. This observation is in agreement with the idea that gradients of auxin determine phyllotaxis (figure 6.8B). However, the existence of auxin gradients remains to be confirmed either by direct auxin measurements or by using auxin-inducible markers.

6.9 A model for the role of auxin transport in phyllotaxis

How could dynamic auxin gradients be generated in a pattern consistent with phyllotaxis? Auxin is known to induce the formation of vascular tissues. The vasculature, in turn, is the route of auxin transport. These findings led Sachs (1991a and b) to propose the ‘canalization hypothesis’, which assumes that in a tissue in which auxin is initially uniform, small random differences in auxin synthesis, transport or metabolism lead to uneven distribution of auxin (figure 6.9). In cells that experience elevated levels of auxin, its capacity to

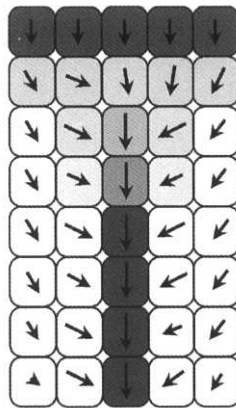


Figure 6.9 Determination of vascular strands by auxin according to the ‘canalization hypothesis’ Auxin producing cells (top row of cells) deliver auxin to neighboring cells, which initially experience equal auxin levels (represented by the second row of cells from top). Small random differences in auxin synthesis, transport or metabolism lead to uneven distribution of auxin. In cells which experience elevated levels of auxin, the capacity to absorb auxin and to transport it along the direction of pre-existing polarization is increased (cell in the center of the third row of cells). This leads to further accumulation of auxin in these cells. The resulting positive feedback mechanism leads to the confinement of the transport routes to narrow files. Auxin is accumulated in these cells (dark cells in the lower half of the central column of cells) and determines that they differentiate into vascular tissues. (Drawn according to models from Sachs, 1991a and b.)

absorb auxin and to transport it along the direction of pre-existing polarization is increased. This leads to further accumulation of auxin in these cells. The resulting positive feedback mechanism, referred to as canalization, leads to the confinement of the transport routes to narrow files, which become determined by auxin to differentiate into vascular tissues. This mechanism is thought to determine the vascular patterning in leaves (Sachs, 1991a and b; Berleth and Mattsson, 2000; Berleth *et al.*, 2000). If vascular differentiation starts from a pre-patterned situation due to the existence of a source or a sink of auxin, then the direction of the developing vasculature is dictated by the pre-pattern. In general, auxin sinks 'attract' vascular strands, and similarly, auxin sources induce the differentiation of vasculature in their vicinity (Sachs, 1991a and b).

Auxin transport has been studied extensively in roots. Auxin is thought to be transported to the root tip through the central stele. In the root tip, auxin is redistributed to the flanks of the root, and then transported back (towards the elongation zone) in the epidermal and cortical cell files (Jones, 1998). Normally, auxin redistribution in the root tip is equally efficient in all radial directions. However, if the root is gravistimulated, auxin flux is preferentially redirected to the lower side of the root with respect to the gravity vector. The higher accumulation of auxin in the lower half of the root inhibits root elongation on this side, leading to curvature and gravitropic reorientation of root growth (Davies, 1995).

One can speculate that acropetal transport in the shoot apex and lateral redistribution of auxin within the meristem could be the basis of phyllotaxis. Auxin produced in maturing leaves could be delivered to the stem and acropetally transported to the meristem in a way analogous to transport in the stele of the root. In the meristem, it becomes laterally redistributed by a PIN-dependent pathway that involves the youngest pre-existing primordia. These primordia could influence the distribution of auxin by absorbing auxin from the adjacent meristem tissues in a way analogous to canalization. Thus, the young primordia would act as sinks of auxin, and in the meristem, sufficient auxin to promote new organ formation could only be accumulated at some distance from the preformed primordia (figure 6.10). As soon as a certain level of auxin accumulation is reached at this position (I_1), organ formation would be induced, and at the same time the induced cells would gain sink capacity. This provides the opportunity for them to participate in the determination of the position of the next primordium. Such a reiterative mechanism could create the phyllotactic patterns found in nature. If only the youngest primordium (P_1) absorbs auxin, leaves would always be initiated at 180° from each other, as is the case in distichous phyllotaxis (figure 6.10A). If the two youngest primordia (P_1 and P_2) compete for auxin, but the ability to absorb auxin declines with increasing age of the primordia, and P_1 has the strongest effect, then new primordia would be placed between P_1 and P_2 , but closer to the latter, as is the case in spiral phyllotaxis (figure 6.10B). If the range of efficient auxin withdrawal by primordia is smaller than half the meristem diameter, two leaves could be

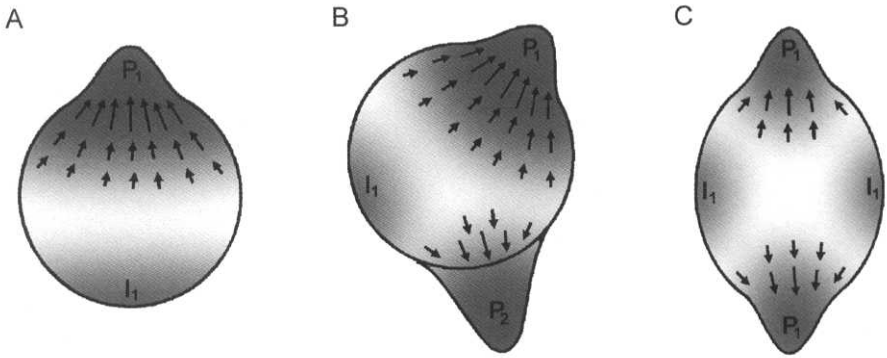


Figure 6.10 A model for the role of polar auxin transport in phyllotaxis. The figure shows three schematic transverse sections through meristems of a distichous (A), a spiral (B) and a decussate plant (C), respectively. The sections are at the level of the youngest primordia (P_1). Primordia are envisaged to absorb auxin (represented by darker shades) from the meristem by a mechanism involving polar auxin transport, thus acting as sinks for auxin. They determine the distribution of auxin, which is acropetally transported to the meristem from the stem. Only at a certain distance from absorbing primordia, can auxin accumulate to levels sufficient to promote leaf formation (I_1). A: Distichous phyllotaxis—in this system, only the youngest primordium (P_1) absorbs auxin, whereas P_2 has either lost the capacity to absorb auxin or it is too remote from the meristem. Therefore, I_1 is always initiated opposite to P_1 . B: Spiral phyllotaxis—if the two youngest primordia (P_1 and P_2) compete for auxin, but the ability to absorb auxin declines with increasing age of the primordia (and with increasing distance from the meristem), then P_1 has the strongest effect. Thus, the next primordium will be placed between P_1 and P_2 , but closer to the latter. C: Decussate phyllotaxis—here, the range of efficient auxin withdrawal by primordia is smaller than half the meristem diameter. Hence, two leaves can be initiated at a time (I_1). Due to mutual competition for auxin, they will be placed at opposite positions, and the resulting leaf pairs will determine future leaf pairs to be initiated with a divergence angle of 90° .

initiated at a time. Due to a mutual competition for auxin, these leaves would be placed at opposite sites, and the resulting leaf pairs would determine future leaf pairs to be initiated with a divergence angle of 90° , as is the case in decussate phyllotaxis (figure 6.10C).

In this context, it is interesting to note that both the auxin transport protein, *PIN1*, and the auxin response transcription factor, *Monopteros*, are upregulated early in organ initiation, before a bulge is visible (figure 6.4D, E, H; Hardtke and Berleth, 1998; Vernoux *et al.*, 2000). Therefore, auxin, as well as auxin transport capacity might be present very early in incipient primordia. Although the youngest primordia (P_1 and P_2) are probably incapable of auxin biosynthesis, and may function as auxin sinks, they will, at some point, start to produce auxin and deliver it to the stem, thus acting as auxin sources. Hence, phyllotaxis could be influenced both by the range of auxin absorption around the youngest primordia and by the timing of the sink-to-source transition of older primordia.

To determine how pre-existing primordia affect auxin distribution within the apex, it will be important to measure auxin levels in the different apical tissues. Furthermore, it has to be established at which stage primordia start to produce

auxin, and to determine the direction of auxin fluxes between primordia of different developmental stages and the meristem, and within the apical stem just below the meristem.

6.10 How does auxin regulate growth in the meristem?

First, it should be noted that, by definition, to grow means to increase in volume. This cannot be achieved in the absence of cell expansion. Cell division alone only leads to smaller and smaller cells with no net growth, as in early embryogenesis of amphibians (Slack, 1991). In contrast, growth could principally take place in the absence of cell division, simply by expansion of existing cells, as in the development of the unicellular trichomes in *Arabidopsis* (Hülkamp *et al.*, 1994). It is obvious that the formation of a primordium requires the coordination of cell division and cell expansion. However, mechanistically, cell division and cell expansion can be linked in different ways. The primary event in organ initiation could be the activation of the cell cycle, followed by cell expansion and differentiation of the proliferating cells. Alternatively, growth could be induced by increased tissue expansion followed by insertion of cross walls to subdivide the increasing cell volumes. While both mechanisms of growth occur in plants (Kaplan and Hagemann, 1991; Jacobs, 1997), the degree to which both operate in the SAM is not clear.

How could auxin induce organ initiation? That is, what are the downstream processes that are initiated by auxin? Auxin is known to regulate various cellular responses, including cell division and cell expansion (Davies, 1995). In the induction of lateral roots, auxin induces cell division, presumably by direct activation of the cell cycle in pericycle cells (De Veylder *et al.*, 1999). In the gravitropic response, auxin is thought to act by regulating differential tissue expansion in the absence of cell division (Davies, 1995). Thus, it appears that the cellular responses to auxin depend on the tissue context and on the developmental stage. Most tissues in which auxin effects have been studied are differentiated tissues. In contrast, little is known about the regulation of cell growth in the meristem and the cellular responses of meristematic tissues to auxin.

Cell division activity in the meristem and in developing primordia can be determined quantitatively by counting the number of dividing cells (using mitotic figures or diagnostic cell cycle genes) and recording the orientation of newly formed cross walls (Laufs *et al.*, 1998b, Lyndon, 1998). Quantitative analysis of cell division activity in *Arabidopsis* floral apices has shown that division rate was increased at the site of incipient flower formation, indicating that induction of the cell cycle may be one of the first signs of growth (Laufs *et al.*, 1998a).

Similarly, in vegetative meristems of *Pisum*, the cell cycle was accelerated at the site of incipient leaf formation (Lyndon, 1998). However, not only the

rate but also the orientation of cell division changed. An increased proportion of cells divided periclinally, that is, the new cell walls were aligned parallel to the meristem surface. Since in plants, new cell walls are usually orientated perpendicular to the direction of growth (Lyndon, 1990), this could indicate that cell division responds to increased outward expansion, instead of being the cause of outgrowth. Indeed, Lyndon (1998) pointed out that ‘the occurrence of periclinal divisions does not, and cannot, cause outward growth’.

Although the role of cell division and expansion at the earliest stages of organ formation is not clear, a number of studies provide indirect evidence that organ formation may be regulated at a supercellular level, possibly by differential regulation of tissue expansion rather than at the level of cell division. These include:

- In wheat seedlings that were γ -irradiated to block cell division, the meristem produced bulges that resembled leaf primordia. These bulges were induced by directional cell expansion of the tunica cells (Foard, 1971).
- Leaf formation in transgenic tobacco plants with reduced cell cycle activity occurred with similar rates as in control plants, and the final size of the leaves was normal. However, cell number was decreased and cell size was increased (Hemerly *et al.*, 1995).
- In maize plants with the *tangled* mutation, the orientation of cell division is deregulated, resulting in an uneven leaf surface. However, mutant plants are similar to wild-type siblings, especially in terms of phyllotaxis and overall leaf shape (Smith *et al.*, 1996).
- In the *Arabidopsis tonneau/fass* mutant, cell division is completely irregular. Although the dimensions of the plants are abnormal, they contain all the tissues at normal positions (Torres-Ruiz and Jürgens, 1994; Traas *et al.*, 1995).

6.11 A role for expansin and the cytoskeleton in organ initiation

If local tissue expansion drives organ initiation, one of the earliest stages in organogenesis should be the induction of agents that induce cell expansion. Expansins are cell wall proteins that are implicated in cell expansion due to their ability to increase cell wall extensibility *in vitro* (McQueen-Mason and Cosgrove, 1994, 1995) and in cell cultures (Link and Cosgrove, 1998). Therefore, they are thought to control cell growth *in planta* (Cosgrove, 1997, 2000). In *Arabidopsis*, tomato and rice, expansins are encoded by gene families comprising several dozen members that are differentially expressed. It is thought that differential expression in the tissues allows precise fine-tuning of cell wall extensibility in space and time. In general, expansin expression coincides with growing tissues, with their induction occurring under conditions that promote

growth (Keller and Cosgrove, 1995; Cho and Kende, 1997; Brummel *et al.*, 1999a; Caderas *et al.*, 2000; Catala *et al.*, 2000). In transgenic *Arabidopsis* plants overexpressing an expansin gene, leaf growth was enhanced (Cho and Cosgrove, 2000). However, in some cases, expansin expression was not correlated with growth (Rose *et al.*, 1997; Caderas *et al.*, 2000), indicating that expansins may also function as modulators of cell wall properties in the absence of cell wall extension (Cosgrove, 2000). It has been shown that an expansin which is specifically expressed in tomato fruits, contributes to tissue softening in ripening fruits (Brummel *et al.*, 1999b).

Since expansin regulates growth of developing tissues, it was of interest to see whether applied expansin was also able to control growth in the undifferentiated cells of the SAM. Expansin protein was applied locally to the flank of tomato meristems at a radial position at which primordium formation was not expected (I2). This treatment induced ectopic organogenesis, indicating that promoting tissue expansion is sufficient to drive morphogenesis (figure 6.11a; Fleming *et al.*, 1997, 1999). It was therefore postulated that local cell wall softening could be the critical step in the initiation of primordia. Although expansin-induced primordia exhibited dorsoventrality and expressed markers for leaf identity (Fleming *et al.*, 1997, 1999), they never developed a vascular system and, therefore, could not grow beyond the stage of a young primordium. This is in contrast to leaves induced by auxin (see section 6.8), which did grow to

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Figure 6.11 A role for expansin in primordium formation at the shoot apical meristem (SAM). A: Expansin protein was applied to the flank of a tomato meristem. This treatment induced the formation of a leaf-like structure with trichomes. Although such primordia exhibit dorsoventrality and express markers for leaf identity, they never develop into mature leaves. (Reproduced with permission from Fleming *et al.*, 1997.) B: *In situ* hybridization of a tomato apex at the level of the youngest leaf primordium (P₁). The next oldest primordia (P₂ and P₃) are visible in clockwise phyllotaxis. The section was hybridized with a probe for *LeExp18*, a tomato expansin gene. At the site of incipient leaf formation (arrowhead), *LeExp18* is upregulated (indicated as a bright signal). (Reproduced with permission from Reinhardt *et al.*, 1998.)

normal sizes and had all the tissues of normal leaves (Reinhardt *et al.*, 2000). This suggests that expansin induces only a subset of events required for proper leaf formation, whereas auxin induces the whole program.

The ability of exogenous expansin to induce primodium formation prompted the authors to look for endogenous expansin genes that could be involved in leaf formation. Indeed, a tomato expansin gene, *LeExp18*, was identified that is expressed in meristematic tissues. *LeExp18* is upregulated at the site of incipient leaf formation (figure 6.11B; Reinhardt *et al.*, 1998), and induction of the gene occurs very early in the induction of leaf primordia before any sign of organ formation can be observed histologically. Interestingly, *LeExp18* expression in the meristem is induced by exogenous auxin (Reinhardt and Kuhlemeier, unpublished results). Thus, *LeExp18* may be a primary target of auxin in organ initiation, and its expression pattern may reflect elevated auxin levels at I_1 . Together, these results suggest that expansin is likely to regulate organogenesis at a very early stage, possibly by acting as the initial trigger of local growth.

Cell expansion is intimately linked with the cytoskeleton (Gunning and Hardham, 1982; Cyr, 1994). The orientation of cortical microtubules predicts the orientation of cellulose microfibrils, and this determines the direction of cell expansion. Although direct proof is missing, the dynamic cortical microtubules are thought to determine the orientation of cellulose microfibrils by guiding the cellulose synthase complex along the plasmalemma (Cyr, 1994). The orientation of microfibrils then dictates the direction of expansion, since the cell wall can most easily expand in the direction perpendicular to the microfibrils. This scenario appears to be true for unidirectional expansion in cylindrical cells with predominant hoop-reinforcement (Green, 1984). If localized tissue expansion is the primary event in organ initiation, then changes in the cortical microtubule arrangement may be useful markers to study the earliest steps in organogenesis.

The orientation of microfibrils in the meristem L_1 -layer has been studied extensively by Green and co-workers (Green, 1986; Green, 1988; Jesuthasan and Green, 1989), who observed that the pattern of microfibrils in the meristem L_1 layer corresponds with the patterns of local tissue expansion during leaf initiation (figure 6.12). Green concluded that undirected internal pressure in the meristem could be translated into directional expansion merely by spatial and temporal fine-regulation of cellulose deposition in the L_1 layer (Green, 1994). Although this theory offers an elegant mechanism to explain the regulation of differential growth, it does not explain the patterning mechanism that regulates microfibril orientation. Interestingly, auxin has been reported to induce reorientation of cortical microtubules (Nick *et al.*, 1992; Shibaoka, 1994). Thus auxin could act both by increasing cell wall extensibility through expansin induction, and influence the direction of expansion through reorientation of microtubules.

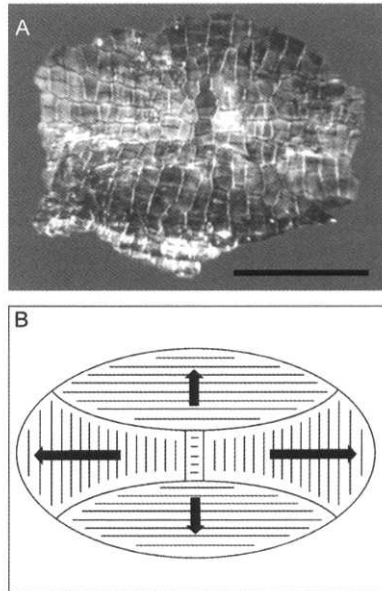


Figure 6.12 The orientation of cell wall microfibrils predicts the pattern of growth in the meristem of *Vinca major*. A: In a superficial (paradermal) section of a meristem of *Vinca major*, the orientation of microfibrils in the L_1 layer can be seen in polarized light. Areas in which the microfibrils are arranged in N-S orientation appear bright. In darker areas, the microfibrils are arranged in W-E orientation. *Vinca major* is a decussate plant. In the specimen analysed, the next pair of leaves would have been formed to the left and to the right. (Reproduced with permission from Green, 1985.) B: Schematic representation of the reinforcement patterns seen in (A). Horizontal and vertical lines represent the orientation of the microfibrils. Arrows indicate the direction of growth, as determined by the reinforcement pattern. Growth will preferentially be directed towards the sites of leaf formation (long arrows).

6.12 Conclusions

The regularity of the spatial arrangement of leaves, flowers and floral organs, has amazed people since the first descriptions of phyllotactic patterns. Phyllotaxis has evoked numerous models to explain the mechanisms that regulate pattern formation in the meristem. During past decades, tremendous progress has been made in understanding meristem establishment and maintenance, as well as the process of organ initiation. However, the molecular and cellular mechanisms that regulate phyllotaxis are only now beginning to unfold.

Whereas originally, biophysical and biochemical models of phyllotaxis have been considered to represent opposing mechanisms, it now appears that they may converge to a coherent model of phyllotaxis with auxin at the center. Evidence is accumulating that patterning mechanisms involve molecular signaling between cells within the meristem. Examples include the signaling system involving

the CLAVATA proteins in the regulation of the stem cell population (Fletcher and Meyerowitz, 2000), or auxin in the patterning of the meristem and organ initiation (Kuhlemeier and Reinhardt, 2001). On the other hand, downstream mechanisms that realize the patterns may well be based on biophysical principles, e.g. modulation of the cytoskeleton arrangement and of cell wall properties. In the future, the combination of genetics with pharmacological approaches and micromanipulation is expected to allow deeper insights into the determinants of phyllotaxis.

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7 Axial patterning and meristematic growth in leaves

Bruce Veit and Toshi Foster

But what is the Leaf? Long and Oft have I sought an answer to this question, without finding anything with which I could satisfy you and myself.

(Speighel, A., 1606 as quoted by Arber, 1950)

7.1 Introduction

7.1.1 Origin of the leaf

The evolution of the leaf marked a significant milestone in the history of land plants, by greatly extending the range of plant architecture (Niklas, 2000). Compared to the stem, whose cylindrical cross-section best serves supportive and conductive functions, the leaf was a triumph for photosynthesis, its laminar form maximising the exchange of gases and capture of light. Simultaneously, the gravity of a non-aquatic environment imposed additional design constraints that gave the leaf clear advantages, its mass increasing only linearly with its surface, in contrast to the greater burdens imposed by the stem. The leaf has also proved a versatile structure, assuming a multitude of shapes and forms over the course of evolution to better exploit prevailing light, wind and canopy conditions. Finally, the leaf has been a point of departure for the evolution of organ types whose structure would serve a variety of novel functions, including storage, support, protection, digestion, reproduction and even advertising.

Despite the significance of the leaf in the evolution of land plants, its origin remains obscure. Fossilized plants from some 400 million years ago suggest that the earliest land plants consisted of a series of naked, dichotomously branched stems. By the end of the Devonian epoch, leaf-like forms had begun to emerge as seen in fossilized material classed within primitive horsetail, fern and seed plant groups (Gifford and Foster, 1988). While it is unclear how the leaf might have been fashioned to clothe the naked stems of these ancient forms, two plausible hypotheses have been offered. The 'Enation' theory (Bower, 1935) supposes that at least one class of early leaf first appeared as flattened avascular outgrowths on the cylindrical stem axis. The simple-formed, single-veined microphyll, as found in the Lycopods, is easiest to accommodate within this framework. More challenging are the complex-veined, megaphyll-type leaves

found in ferns and seed plants, which have been more commonly modelled as modified complex branch systems. For example, the 'telome' theory (Zimmermann, 1953) supposes that the leaf originated from conversion of a series of dichotomously branched stems through a process of 'planation', in which branches became reoriented into a single plane, followed by 'webbing', in which laminar tissue extended out to join adjacent branches.

7.1.2 Relationships between mechanisms controlling growth in the leaf versus stem

This chapter attempts to address the origin of the leaf by considering its essential nature, especially those aspects that relate to its pattern of growth and development. If the leaf was in fact fashioned through the progressive modification of a branched shoot system, as supposed by the telome theory, some evidence for this origin might be found in the nature of mechanisms that control its growth and development. How similar are these mechanisms to those that control the growth of the stem? Would the occurrence of common mechanisms suggest anything about how the leaf first evolved? Some insights into these questions have been offered by the advent of molecular genetics. With this perspective, the concept of homology has taken on added meaning, with linkages inferred by morphological reasoning now finding support through a detailed knowledge of the molecular mechanisms that underpin them. Goethe's proposition that the leaf and floral organs are homologous has found much favour within this new framework. Will this perspective reveal comparable fundamental relationships between the leaf and stem?

Given the central role of meristematic tissues in the formation of many elements of the plant body (see chapter 1 of this volume), it is reasonable that a focus on the nature of these tissues should lead this line of inquiry, including the question, 'To what extent can meristematic patterns of growth in the leaf be compared to those of the stem?' Increasingly sophisticated approaches, including clonal analysis and the use of markers for mitotic activity, have been brought to bear on this question to provide a more dynamic view of plant development. At the same time, molecular genetic approaches have introduced a complex repertoire of players and defined their roles and even dialogues. Whether the growth and development of leaves and shoots are regulated by similar mechanisms, and therefore might be considered homologous at some level, forms a central theme for this chapter. This issue is addressed by first reviewing basic patterns of growth in the developing leaf, attempting to define instances that involve regulated patterns of cell division and growth. The role of axial patterning processes in determining such growth patterns is examined in detail, together with their molecular bases. Speculation is offered on mechanisms by which these growth processes might be initiated and coordinated in a position-dependent manner. Within this framework, the evolutionary origin of

the leaf is considered, with discussion of future investigations that might clarify its relationship to the stem.

7.2 A working definition for ‘meristematic tissues’

7.2.1 The SAM: apically limited patterns of meristematic growth

To evaluate the role of meristematic tissues in the developing leaf, it is first necessary to discuss the criteria and utility for defining tissues as such. A broad definition might include any spatially coherent grouping of cells whose mitotic behaviour differs from that of surrounding tissue. As discussed in chapters 1 and 2 in this volume, perhaps one of the most clear-cut examples of such a tissue would be the shoot apical meristem (SAM). Undifferentiated ‘initials’ located at the centre of the apex undergo infrequent polarized divisions to produce two classes of mitotic derivatives. Those cells at the tip remain undifferentiated and slowly dividing, thus preserving a population of initials, while those displaced away from the tip divide more frequently, producing clones of cells that ultimately differentiate.

The SAM provides a clear example of the heuristic value of the concept of ‘meristematic tissues’, owing its persistence to the regulation of both the frequency and polarity of cell divisions across the 3-dimensional space of the shoot apex. Although it is unclear how these coordinated patterns of cell division are regulated, a detailed knowledge of the characteristic division patterns within the SAM has sharply focused attention on the nature of mechanisms by which these patterns are maintained. As discussed in chapter 2, lineage and surgical analyses indicate that positional-dependent cues are likely to play a major role, while more recently, molecular genetic approaches have identified regulatory genes that are likely, in some way, to generate and interpret these cues.

7.2.2 Meristematic growth in the leaf: dynamic patterns in two dimensions

Whether analogous mechanisms operate in the context of the developing leaf is less clear. Unlike the shoot, in which the persistence of initials in an apical position simplifies their analysis, the more complex and dynamic pattern of leaf development has complicated efforts to define meristematic tissues and characterize their behaviour. Compared to the shoot apex, the geometry of the leaf primordium undergoes profound changes during its ontogeny (Lyndon, 1998; see also figure 7.1). Programmes of meristematic growth sweep across and transform the leaf as it emerges from the stem. While apical meristems persist through multiple cycles of lateral organ initiation, the leaf generally has a more limited and determinate pattern of development, in which meristematic regions are ultimately consumed by terminal differentiation. Nevertheless, like

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Figure 7.1 Schematic representation of the development of the basic axes of the leaf. Abbreviations: init, initial; prim, primordium. (Waites and Hudson, 1995, *Development*, 121; reproduced with permission of the Company of Biologists Ltd.)

the shoot, leaves display highly stereotyped patterns of development that can be characterized by spatially defined patterns of regulated cell division and expansion. Whereas in the shoot apex, this regulation is evident in the maintenance of a discrete population of apical initials (see chapter 2 of this volume) and the periodic emergence of lateral organs at predictable sites (chapter 6), in leaves, such regulation leads to the elaboration of characteristic shapes and forms. By analogy with the SAM, the first step towards understanding the mechanisms that underlie the development of the leaf requires a clear description of process itself, with particular attention to the timing and localization of meristematic growth.

7.3 Leaf ontogeny

7.3.1 The determination of the leaf

To fully describe the patterns of cell division associated with the developing leaf, it is necessary to discuss at what point its development actually begins. As discussed by Lyndon (1998), the first unequivocal outward sign that leaf development is underway can be equated with appearance of a bulge on the flank of the shoot apex. This change also provides a baseline for describing the developmental age of the leaf in terms of the plastochron (P), a unit corresponding to the time interval between successive leaf initiation events (Esau, 1965). This chapter adopts the convention in which the group of founder cells, or anlagen of the leaf, are collectively referred to as the P0 primordium at the point in time when the leaf primordium first becomes geometrically distinct from the SAM. Recognizing the regular time intervals between successive leaf initiation events, the next older primordium is designated P1, while the anlagen of leaves that have yet to emerge are described by negative numbers (e.g. P-1 to describe the anlagen of a leaf 1 cycle before it is first evident).

Although a detailed account of factors that influence the position of leaf initiation is presented in chapter 6 of this volume, a brief summary relating to when the leaf is first conceived is offered here. Surgical experiments provide evidence for the inception of the leaf some time before it first emerges. In both angiosperms and ferns, radial incisions or punctures to the SAM disturb the normal phyllotaxy, suggesting that cells on the flanks of the apical dome become determined in a manner influenced by positional information (reviewed in Steeves and Sussex, 1989). By contrast, similar treatments on more proximal regions of the apex have little effect on the positioning of leaves, suggesting that these cells have somehow become determined in a way that distinguishes them from their uncommitted neighbours. Taken together with clonal analyses (Poethig, 1987), a consistent picture emerges in which the leaf is organized from a group of several dozen cells from outer layers of the apical meristem.

7.3.2 *Patterns of cell division associated with the inception of the leaf primordium*

Given the physical context in which the leaf arises, it is reasonable to consider the significance of cell division to the earliest stages of its development. Leaf primordia first emerge at a relatively fixed distance from the shoot apex, a phenomenon that has been linked to the 'zonation' of the shoot apex (reviewed in Steeves and Sussex, 1989; Lyndon, 1998; see also chapter 2 of this volume). Slowly dividing cells in the 'central zone', including apical initials, are encompassed by the ring-shaped 'peripheral zone' of more rapidly dividing cells, including sites at which leaf primordia first emerge. Since this region, sometimes referred to as the *anneau initial*, includes both sites at which the leaf primordia emerge as well as laterally flanking tissues, it would seem that additional factors act to determine the radial positioning of leaf primordia. Chapter 6 in this volume offers a more detailed discussion of these, suggesting that such factors may act independently from processes that prevent emergence of organ primordia close to the tip. However, the notion that higher rates of cell division may be a prerequisite for organ formation is reinforced by changes that occur upon flowering, in which the emergence of floral organ primordia higher on the apical dome coincides with increased rates of cell division in this region (Steeves *et al.*, 1969).

Changes in the polarity of cell division are also associated with early stages of leaf initiation, particularly in tunica layers where departures from a consistently anticlinal pattern of division are most apparent (Esau, 1965). This shift could be interpreted to indicate an active process that reorients division of cells in the future leaf primordium to planes that are parallel to its axis of growth. Alternatively, this same outcome might result from a localized relaxation of factors that would otherwise maintain a pattern of anticlinal division. Observations in pea favour the latter model by showing that division planes in regions corresponding to the incipient leaf primordium are randomly oriented in 3-dimensional space

(Lyndon, 1972). Modest transient increases in the rate of cell division are also associated with presumptive sites of leaf initiation (reviewed by Lyndon, 1998).

Changes in the frequency and polarity of cell division in regions corresponding to the incipient leaf primordium raise the issue of whether these processes drive the formation of the new growth axis of the emerging leaf, versus being a passive consequence of this growth. Classic studies show that leaf-like structures can still emerge from the SAM of wheat seedlings after irradiation to inhibit mitotic division, suggesting that the establishment of the leaf growth axis does not depend on cell division per se (Haber, 1962; Foard, 1971). It has been suggested that these types of morphogenetic events are directed by supracellular processes, which coordinate the polarity of growth. Thus, rather than serving to nucleate new growth centres, division may simply maintain a consistent level of cellular partitioning demanded by metabolic and structural factors (Green, 1976; Kaplan and Hagemann, 1991). On the other hand, the emergence of leaf-like structures in the absence of cell division in plants may depend on polarities that are established through division-related processes, but whose normal culmination by cytokinesis has been blocked by irradiation.

In pea, measurement of mitotic indices across the SAM show that localized increases in the frequency of cell division corresponding to incipient leaf primordia precede any distortion of the apical dome (Lyndon, 1970), suggesting that cell division associated with the earliest phases of initiation is not simply a passive consequence of volume increase. In light of recent data, which show that localized increases in the extensibility of tissue are also associated with the emergence of leaf primordia, it is conceivable that increases in the rate of cell division could be triggered by subtle differences in strain or pressure across the meristem. Studies in tomato illustrate how such differences might arise by showing the localized expression of genes encoding expansins, proteins that act on cell walls to increase their extensibility (Reinhardt *et al.*, 1998). Thus, as previously conjectured, increased rates of cell division could be an indirect outcome of mechanisms that induce localized changes in the mechanical properties of the SAM (Green, 1987, 1999). Alternatively, localized increases in division might instead reflect phyllotactic signals that act directly on cells within the leaf anlagen to decrease their cycling times.

7.3.3 *Molecular markers for leaf founder identity*

Further insight into processes that mediate the early development of the leaf has been gained through the characterization of a closely knit family of homeodomain genes, referred to here as '*KN1*-like' genes, whose expression patterns anticipate the formation of morphologically defined boundaries (Jackson *et al.*, 1994). The founding member, *KNOTTED 1* (*KN1*) of maize, is expressed at high levels throughout the shoot apex except in regions corresponding to putative leaf founder cell populations (Smith *et al.*, 1992; see also chapter 2 of this

volume). Ironically, the gene was first known by its dominant forms whose ectopic expression in leaves distorts the pattern of lamina growth, causing the formation of 'knots' (Hake and Freeling, 1985). Indeterminate growth is also seen when *KN1* genes are ectopically expressed by fusion to 35S promoters (see section 7.6.3). Conversely, loss of function mutants have been described for *KN1*-like genes, including *SHOOTMERISTEMLESS (STM)* from *Arabidopsis* (Long *et al.*, 1996) and *KN1* itself (Kerstetter *et al.*, 1997; Vollbrecht *et al.*, 2000) in which the SAM is either absent or poorly maintained. Thus, *KN1*-like genes appear to promote indeterminate forms of meristematic growth.

Given the spatial and temporal pattern of the downregulation of *KN1*-like genes, it is tempting to speculate that their downregulation acts in some way to define founder cell populations, and hence plays a key role in maintaining the regular phyllotaxy of the plant. However, in cases where *KN1*-like genes are artificially expressed in a constitutive manner, no obvious changes in phyllotaxy are seen, suggesting that the complete absence of *KN1*-like gene expression is not a prerequisite for establishment of founder cell identity. In tobacco, however, overexpression of an endogenous *KN1*-like gene, *NTH1*, increases the plastochron ratio, suggesting an inhibition of the rate of leaf initiation relative to the radial growth of the shoot apex (Tamaoki *et al.*, 1999). However, conclusions of these overexpression studies must also be qualified in that typically only a single *KN1*-like gene is overexpressed, whereas normally more than one is downregulated in the pre-emergent leaf primordium. Thus, it remains possible that the downregulation of an ensemble of *KN1*-like genes contributes to the determination of founder cell populations, but in a way not easily perturbed by overexpression of single genes in this class.

In contrast to the *KN1*-like genes, which are downregulated in founder cells of the leaf, some regulatory genes are activated in these regions. The *PHAN* gene of *Antirrhinum*, encoding a MYB family transcription factor, is expressed throughout the anlagen of young primordium from P0 onwards, but is not detectable elsewhere in the shoot meristem (Waites *et al.*, 1998). As is the case for *KN1*-like genes, it is unclear whether *PHAN*-like genes play a pivotal role in the establishment of leaf founder cell populations. Given that *PHAN* is specifically expressed in this population of cells and that in its most extreme form, *phan* mutants are blocked in the formation of leaf primordia, it is tempting to equate *PHAN* activity with the determination of founder cell identity. However, the block in the formation of leaves has been interpreted to be a secondary consequence of a failure of the SAM to maintain itself. The authors suggest that the primary function of *PHAN* is to promote the establishment of an adaxial cell identity in the developing leaf, which in turn promotes the proliferative activities of the nearby SAM (discussed more fully in the next section).

A third class of regulatory gene which may influence the selection of leaf founder cell populations is represented by the RNA-binding protein encoding gene, *TERMINAL EAR 1 (TE1)* of maize (Veit *et al.*, 1998). Its loss-of-function

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Figure 7.2 Pattern of mRNA accumulation for the *TERMINAL EAR 1* gene of maize. A: Transverse cross-section showing semicircular ring of mRNA accumulation bracketing the P0 primordium. B: Longitudinal section showing alternating bands of TE1 mRNA accumulation. C: Schematic diagram summarizing mRNA expression patterns. (Veit *et al.*, 1998, *Nature*, **393**, reproduced with permission of MacMillan publishing.)

phenotype includes changes in the pattern of leaf initiation, including an increase in the rate of leaf initiation with resulting leaves positioned abnormally along the shoot axis. Consistent with a possible role in defining founder cell populations. *TE1* is normally expressed, beginning with P-1, in a series of semicircular rings that bracket sites at which leaves first emerge (Figure 7.2). In the loss-of-function mutant, it is hypothesized that these initiation events become less constrained, leading to precocious leaf initiation events and changes in phyllotaxy. Because the expression domains of *TE1* overlap with regions that are eventually incorporated into the margins of the maize leaf, this model would assume that the early phases of leaf initiation, during which the position of the primordium becomes fixed, are somehow distinct from subsequent stages, during which marginal domains become established. Support for the existence of distinct lateral domains within the incipient leaf and the progressive recruitment of founder cell populations in lateral domains is found in the analysis of the *narrow sheath* mutants of maize, in which leaves initiate normally but then fail to develop lateral domains (Scanlon *et al.*, 1996; see also section 7.4.3). Associated with this deletion are changes in the pattern of *KN1* expression, in which the normal progressive spreading of the downregulated pattern into lateral regions fails to occur.

7.4 Axial patterning of the leaf

7.4.1 Determination of dorsiventrality

One of the most salient characteristics of the leaf that distinguishes it from the stem is its planar organization (figure 7.1). Related to this architecture is a pattern

of dorsiventral asymmetry, in which the adaxial (facing upwards and closer to the shoot apex, sometimes also referred to as the dorsal surface) and abaxial (ventral) faces of the leaf show distinct patterns of development, including the distribution of cell types (e.g. stomata, trichomes and other specialized epidermal cell types) and cuticular waxes. Differences are also observed in the organization of internal tissues, such as the relative position of vascular elements and palisade versus spongy mesophyll (Esau, 1965).

How this dorsiventral axis of symmetry becomes established is a long-standing question that has been addressed in part through surgical experiments (reviewed in Steeves and Sussex, 1989). In dicotyledonous species, the primordium first emerges as a radially symmetrical peg-like outgrowth on the flank of the shoot apex, later acquiring its mature laminar form through growth extending from the lateral domains of the primordium. Given the context in which this development occurs, an obvious source for signals that might serve as cues to establish dorsiventrality would be the polarized shoot system upon which the leaf is borne. This hypothesis finds support in experiments of Sussex (1955), as well as others (reviewed in Steeves and Sussex, 1989), in which tangential incisions that isolated newly emerged primordia from the apex led to the development of radial symmetrical organs. These results implicate a signal that originates from the apex, which could act in a distant-dependent manner on the upper surface of the leaf to repress an abaxial programme of development or, alternatively, to promote adaxial development.

The nature of mechanisms that establish and maintain dorsiventrality in the leaf has begun to emerge through molecular genetic analysis of mutants in which these processes are compromised. Analysis of the *Antirrhinum* mutant discussed in section 7.3.3, *phantastica*, has offered important insights (Waites and Hudson, 1995; Waites *et al.*, 1998). Under some growth conditions, relatively normal-shaped leaves are formed. Upon closer inspection, however, many of these leaves show patches of tissue with abaxial characters on their adaxial surface. In extreme forms, the leaf primordium is initiated normally, but subsequent growth leads to the formation of a radially symmetrical organ in which adaxial cell types are totally absent (figure 7.3). On the basis of the linkage between the absence of adaxial patterning and the failure of lamina development, Waites and Hudson (1995) proposed that, in normal leaves, the juxtaposition of adaxial and abaxial tissues induces the formation of lateral domains of the leaf. This model receives additional support from detailed analysis of mutant leaves in which leaf margin-like ridges are often observed along the boundaries of ectopic abaxial tissue. Radialized leaves were also associated with another intriguing phenotype in which the SAM activity is shut down, suggesting that the continued normal activity of the SAM may require some form of ongoing communication with dorsiventrally patterned leaves, with adaxial tissues promoting SAM activity.

While the precise mechanism by which *PHAN* contributes to the dorsiventral patterning of the leaf is unclear, molecular analyses of *PHAN* and its orthologues

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Figure 7.3 Transverse sections through: A) wt and B) *phan* leaves illustrating the lack of dorsiventral asymmetry in mutant leaves. (Waites and Hudson, 1995, *Development*, **121**, reproduced with permission of the Company of Biologists Ltd.)

have provided important clues. As discussed in section 7.3.3, *PHAN* encodes a MYB class transcription factor which is expressed in the anlagen of the leaf primordium prior to its emergence. Genetic analyses of a *PHAN* orthologue from maize, *ROUGH SHEATH2 (RS2)* (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999), suggest that *KN1*-like genes are a target for regulation. Like *phan*, *rs2* mutants exhibit axial patterning defects. The possibility that these defects might be mediated by *KN1*-like genes was first suggested by very similar phenotypes associated with mutations to *KN1*-like genes, which cause their ectopic expression in young leaf primordia (Smith *et al.*, 1992; Jackson *et al.*, 1994; Schneeberger *et al.*, 1995; Foster *et al.*, 1999b; Muehlbauer *et al.*, 1999). These same patterns of ectopic expression are seen both in *phan* and *rs2* mutants, suggesting that these genes normally suppress expression of *KN1*-like genes in young leaf primordia.

While these results suggest that *PHAN*-like transcription factors may normally repress the expression of *KN1*-like genes in the developing leaf, it is unclear whether the ectopic expression of these genes per se is directly linked to defects in dorsiventral patterning. Although *rs2* mutants are disturbed in axial patterning, these defects have been interpreted as primarily affecting the proximal/distal axis, with only subtle changes to the dorsiventral axis. A similar bias towards proximal/distal patterning defects is also evident in phenotypes associated with dominant mutations to *KN1*-like genes. In addition, transgenic studies in which *KN1*-like genes are overexpressed disturb the development of the leaf, but fail to radialize it in a simple manner. It therefore seems possible that *PHAN* normally regulates not only *KN1*-like genes, but also additional factors that may have a more direct role in dorsiventral patterning. The functions of *RS2* and *PHAN* may have diverged, such that in maize factors that regulate dorsiventral patterning are controlled by some other gene. The *leafbladeless (lbl)* mutant of maize could represent one such gene, with mutant leaves showing various degrees of abaxialization and, in extreme cases, becoming totally radialized (Timmermans *et al.*, 1998). Like *rs2* mutants, *lbl* leaves show alterations to the

downregulated pattern of expression of *KN1*-like genes, which are notable in being apparent in very early P0 primordia. The phenotypes of the *lbl* mutant suggest that the gene may normally be required for recruitment of cells into lateral domains of the leaf via a mechanism that involves downregulation of *KN1*-like genes, and that the more pronounced lateral defects of *lbl* compared to *rs2* reflect the early timing of aberrant *KN1*-like gene expression in the pre-emergent leaf primordium.

Genetic analyses have also revealed other players that function in dorsiventral patterning of the leaf. *PHABULOSA*, defined by a semidominant mutation, *phb-1d*, produces radialized leaves that superficially resemble those of *phan* (McConnell and Barton, 1998). Upon closer inspection, however, it is seen that they exhibit an adaxial cell identity in contrast to the abaxialized *phan* leaves. A further difference is the presence of axillary meristems that form around the entire circumference of the leaf base. This association reinforces the model suggested by the *phan* mutants, in which SAM activity is promoted by proximity to tissues having an adaxial identity. In the case of *phb-1d*, the development of meristems in close association with ectopically formed adaxial tissues suggests that an adaxial cell identity in some way promotes SAM activity. Other members of the *PHAB* gene family also appear to promote SAM activity, as suggested by the loss of function phenotypes of the *REVOLUTA* gene, in which axillary meristems fail to form (Talbert *et al.*, 1995). Molecular cloning of *PHAB* reveals that it belongs to a small family of homeodomain genes, many of which show expression in provascular tissues of the stem (Otsuga *et al.*, 2001). The members of this family are also distinguished by the presence of a lipid/sterol binding motif, suggesting that their activity may be modulated by a yet to be identified ligand.

A second class of genes that appears necessary for adaxial patterns of development is defined by the *argonaute1* (*ago1*) (Bohmert *et al.*, 1998) and *pinhead/zwillie* (*pnh/zll*) (McConnell and Barton, 1995; Moussian *et al.*, 1998) mutants of *Arabidopsis*, which produce abaxialized lateral organs. Although the more extreme transformations observed in double mutants suggest some overlap in function, the genes show distinct expression patterns (Lynn *et al.*, 1999). *AGO* is expressed from the globular stage embryo onward, with its mRNA becoming evenly distributed throughout the SAM and associate leaf primordia. *PNH/ZLL* is initially expressed from the four cell stage in the embryo proper, then becoming restricted to provascular tissues and adaxial domains of the cotyledons. In vegetative seedlings, *PNH/ZLL* mRNAs are highly expressed in developing vascular strands, with lower levels detected in the adaxial domains of leaf primordia from P-1. The early expression of *PNH/ZLL* and *AGO* suggests that this class of gene acts early in patterning of the leaf primordia, consistent with the failure of *STM* protein to accumulate in the embryos of double mutants. While the biochemical mechanism by which *PNH/ZLL* and *AGO* functions is unclear, recent studies (Fagard *et al.*, 2000) reveal a requirement for *AGO* in

post-transcriptional gene silencing (discussed more fully in chapters 2 and 3 of this volume), suggesting the intriguing possibility that early patterning of the leaf involves this mode of gene regulation.

Another significant class of gene that functions in dorsiventral patterning is defined by a group of mutants in *Arabidopsis* whose phenotypes are manifest in a range of different lateral organs. The founding member of this group, *CRABS CLAW*, is defined by recessive mutations that lead to a distorted pattern of carpel development (Bowman and Smyth, 1999). Cloning of *CRC* has revealed that it belongs to a group of six zinc finger-type transcription factors, the *YABBY* family. *YABBY* genes have been shown to mediate dorsiventral patterning of the various lateral organs produced by *Arabidopsis* (Siegfried *et al.*, 1999; Bowman, 2000). Double mutant analysis between members of this gene family suggests they act redundantly, to varying degrees, depending on the organ type in question. Thus, in the developing inflorescence, the loss of function mutations to one of these, *fil*, leads to abaxialized filamentous structures. By contrast, single loss of function mutations to other family members *YABBY2* and *YABBY3*, by themselves, have no obvious phenotype. However, when combined with *fil*, both vegetative and floral lateral organs are radialized with only adaxial characters present. Conversely, when *FIL* gene by itself is artificially overexpressed by fusion with a 35S promoter, plants produce cotyledons in which the adaxial surfaces of the cotyledons display characteristics normally associated with the abaxial surface. This abaxialization is also associated with arrested meristem development in a manner that parallels the arrested meristem phenotype associated with abaxialized *phan* leaves of *Antirrhinum*. These observations, together with expression studies, which show the genes are normally expressed on the abaxial surfaces of developing primordia, suggest that members of the *YABBY* gene family normally act to promote an abaxial cell fate. In *PHAB* mutant, the lack of abaxial cell types is associated with the absence of *YABBY* gene expression, suggesting that *YABBY* genes act downstream of *PHAB*.

7.4.2 Proximal/distal patterning and morphogenesis

Although the proximal/distal axis of the leaf first becomes apparent with the emergence of the leaf primordium, precisely how various distinct programmes of development become established along this axis remains unclear. Nevertheless, significant progress towards understanding this process has been made in monocotyledonous systems, especially in grasses such as maize, where mutations exist that alter the positioning of morphologically defined domains along the proximal/distal axis. The maize leaf is typical of most grasses, with four distinct domains arrayed along the proximal/distal axis (Sylvester *et al.*, 1990). Most proximal is the sheath, which completely wraps around the stem along its full length from its point of attachment, thus contributing to the structural integrity of the shoot. The most distal domain is the blade, which tapers from its broad base

to an acute tip. Between the blade and sheath lies the ligule and adjacent auricle region. The ligule consists of a narrow flap of tissue on the adaxial surface of the leaf that is thought to function like a gasket to prevent water from collecting between the sheath and stalk. Distal to the ligule is auricle tissue, consisting of two wedge-like strips of tissue flanking the midrib that function like hinges to orient the blade away from the shoot.

Because of the large size of the maize leaf and the distinct tissue morphologies associated with regions arrayed along its proximal/distal axis, mutations that perturb its normal patterning are easily recognized and, moreover, are generally viable. With this system, Freeling (1992) and Hake and co-workers (1995) have characterized several dominant mutations that disturb the normal patterning along the proximal/distal axis. The *KNOTTED 1* gene (discussed in section 7.3.3), now known for its role in SAM maintenance, was previously described in terms of perturbing normal proximal/distal patterning of the leaf in which ligule tissue develops in ectopic positions on the blade parallel to lateral veins. Analogous phenotypes, in which ligular or sheath tissues appear shifted to more distal positions, are associated with a number of other similar dominant maize mutants, including *ROUGH SHEATH 1* (Becraft and Freeling, 1994), *GNARLEY 1* (Foster *et al.*, 1999b), *LIGULESS 3* and *LIGULESS 4* (Fowler *et al.*, 1996). Subsequent molecular analyses have shown that all of these genes encode homeodomain transcription factors that are closely related to *KN1* (Hake *et al.*, 1995; Reiser *et al.*, 2000). *In situ* hybridization and immunolocalization studies indicate that these genes are expressed in distinct domains within the SAM that appear to demarcate morphological boundaries associated with the incipient leaf primordium, but which are normally downregulated before its emergence. In mutants, this pattern of downregulation is perturbed, with expression arising in the post-emergent leaf.

A series of elegant studies, in which dominant *KN1* class mutants were subjected to mosaic analysis, have afforded significant insights into the possible nature of proximal/distal patterning. In plants in which tissue carrying the dominant mutant allele is juxtaposed with sectors of tissue in which the mutant allele has been lost through x-ray induced chromosome breaks, a sharp boundary is seen, in which tissues within the hemizygous sector have a more distal identity than in adjacent tissue carrying the dominant mutant allele (Sinha and Hake, 1990; Becraft and Freeling, 1994; Foster *et al.*, 1999a; see also figure 7.4). This type of shift has been interpreted in terms of the *KN1*-like genes interfering in a cell-autonomous manner with the normal progression of a 'maturation schedule' (Freeling, 1992). It is supposed that during normal development, the leaf progresses through a stepwise series of competence states, which begins with 'competence to form sheath' and finishes with 'competence to form blade'. This maturation schedule is proposed to begin first in the earliest formed distal parts of the leaf and would begin later in the more proximal regions corresponding to the future sheath. At some point during the leaf's development,

Image Not Available

Figure 7.4 Distortion of proximodistal patterning by the *GNARLEY 1 (GNI)* gene of maize. A: Normal maize leaf showing the ligule (lg) and auricle (a) tissues separating the sheath (s) and blade (b) regions. B: Same region of a *GNI* leaf showing distal displacement of sheath, ligule and auricle tissues into the blade of the leaf. C: Scanning electron microscope (SEM) image of a P4 to P5 leaf showing early stages of ligule formation. D: SEM image of a region similar to (C) from a *GNI* mutant, showing distorted pattern of ligule initiation. (Toshi Foster, PhD Thesis, University of California, Berkeley, 1997.)

a signal sweeps through the leaf, causing regions to differentiate according to the competence state they have attained. For regions in which *KN1*-like genes are ectopically expressed, the progression through competence states is retarded, leading to a ‘younger’, more proximal identity.

One test of this model was provided by a mosaic analysis in which the somatic activation of *LIGULESS4* in the leaf, via a transposon mediated mechanism, provided a large number of sectors whose time of inception could be inferred by their relative size (Muehlbauer *et al.*, 1997). Larger sectors, that would have originated relatively early in leaf development, were associated with the greatest distal displacement of proximal tissue types. On this basis, it was proposed that the proximal/distal patterning of the leaf occurs over an extended period of time.

How the ectopic expression of *KN1* class homeodomain genes would retard the progression through a series of competence states will remain unclear until the targets of these transcription factors are known and how the activation or repression of these genes relates to the establishment of competence states.

It could be argued that similar mechanisms operate during the proximal/distal patterning of dicotyledonous leaves. As discussed in the previous section, loss-of-function mutations to the *PHAN* gene of *Antirrhinum* lead to similar patterns of ectopic expression of a *KN1*-like gene. Although this expression has been linked to defects in the dorsiventral axis, certain aspects of the *phan* phenotype could equally well be interpreted as patterning defects along the proximal/distal axis. In mildly transformed plants, heart-shaped leaves with longer petioles are observed, a phenotype that could be viewed as a distal displacement of proximal characters. For extreme forms of *phan*, the radially symmetrical leaves could be interpreted as consisting entirely of petiole-like structures, though this would not completely account for the radially symmetrical vasculature of the transformed leaf. A modified interpretation that addresses this issue would be that ectopic *KN1*-like expression in *phan* leaf primordia retards the maturation of the leaf to such an extent that its patterning progresses no further than the radial symmetry manifest in the P1 leaf primordium.

The disparity between mechanisms that pattern monocot versus dicot leaves might also reflect fundamental differences in their evolutionary history. On the basis of comparative developmental studies and analysis of serial changes in leaf morphology, Kaplan (1973) concluded that the blade regions of monocots and dicots are not homologous. The blades of dicot leaves are proposed to develop from an 'upper leaf zone'. In monocots, this upper zone remains rudimentary, with the blade region an elaboration of the meristem-encircling, basal zone of the leaf. This example provides a clear example of how comparative morphology can offer an essential conceptual framework for future molecular studies.

The recent cloning and characterization of *ASYMMETRIC 1* (*as1*), the putative *Arabidopsis* orthologue of *PHAN*, suggests that taxon specific factors within dicotyledonous plants may also influence dorsiventral patterning (Byrne *et al.*, 2000). Like *phan* and *rs2* mutants, ectopic expression of *KN1*-like genes is observed in post-emergent leaf primordia; however, like the maize *rs2* mutant, dorsiventral patterning defects are absent. Instead, lobing of leaves is seen that is similar to that conditioned by artificial overexpression of *KN1*-like genes (see chapter 2 of this volume for further discussion).

7.4.3 Lateral patterning

The planar architecture of the leaf as well as its characteristic shape depend on a number of discrete factors that appear to specifically mediate the establishment and differentiation of lateral pattern elements of the leaf. While genetic

factors that mediate these processes remain obscure at a molecular level, genetic studies have offered some insight into how certain elements of lateral pattern become determined. In the typical pattern of angiosperm leaf development, overt development begins the emergence of elements that will eventually form the midrib, with subsequent development of lateral elements. This sequence of development is consistent with the supposed hierarchy of determination suggested by previously described genetic studies, which suggest that lateral development depends on the juxtaposition of abaxial and adaxial tissues. Once triggered, however, lateral development appears to require the activity of additional regulatory elements.

In maize, two such elements appear to correspond to the redundantly acting loci, *NARROW SHEATH 1* and 2, which are necessary for the establishment of lateral domains of the leaf (Scanlon *et al.*, 1996; Scanlon and Freeling, 1998). In their absence, an abnormally narrow leaf develops, in which the lateral domains of the leaf are deleted. Associated with this altered leaf morphology are changes in the normal pattern of *KN1* downregulation in the pre-emergent leaf primordium. The first stages of leaf initiation appear normal, with downregulation of *KN1* occurring in the analagen of the midrib region of the incipient leaf primordium. Subsequently, however, the normal spreading of this downregulated pattern into lateral domains fails. Mosaic analyses of leaves, which contained isolated aneuploid sectors lacking *NS1* and *NS2*, suggest that the two genes act redundantly to propagate signals required for recruitment of cells into the lateral domains of the leaf (Scanlon, 2000). Cloning of the *NS1* and *NS2* loci should provide insights into the nature of this signal and its relationship to the downregulation of *KN1*.

The analysis of leaf shape mutants in tobacco has offered insights into lamina formation in dicotyledonous species. The *lam1* mutant of *Nicotiana sylvestris* initiates leaves normally, with normal formation of abaxial and adaxial domains along the midrib analagen, but fails to elaborate normal lateral domains (McHale, 1993; McHale and Marcotrigiano, 1998). Lateral growth of the lamina is attenuated in the mutant, with a failure of normal adaxial patterning in the lamina tissue that does form. Mosaic analyses of tissues, in which isolated clusters of *lam1* cells assumed a radial pattern of development, suggest that *LAM1* is normally required throughout lamina development. These studies also reveal that the *LAM1* gene product has the ability to support normal dorsiventral patterning in adjacent cell layers from which it is missing, suggesting either an inductive interaction or, alternatively, some limited ability for movement of the *LAM1* gene product.

Interestingly, in leaves where ventralized clusters were juxtaposed with normal leaf tissue, ectopic lamina analogous to those associated with *phan* mutants were not observed. The authors point out that this difference might reflect the fact that the upper epidermis of 'normal' sectors was in fact still genotypically and phenotypically *lam1*, suggesting the induction of blade tissue requires a distinct

dorsiventral boundary extending through all layers. A further observation related to mosaics, in which *LAM1* was missing from the epidermis and the lower and upper mesophyll, was the absence of unique cell types normally associated with the margins of the leaf, suggesting that the determination of these marginal domains may also depend on dorsiventral patterning. The authors suggest that these marginal cell types may represent a neutral zone that blocks further lateral expansion normally associated with juxtaposition of abaxial and adaxial tissues. Consistent with this view are differences in the polarity of cell division in marginal regions, as reflected by variegation patterns of periclinal chimeras, which indicate that all layers of marginal tissues typically derive from L1, in contrast to more internal domains of the lamina that derive from both L1 and L2. Similarly, the polarity of divisions within the plane of the leaf also appears to differ, with clonal sectors in marginal regions running parallel to the leaf edge, in contrast to more internal sectors that tend to run from margin to midrib.

The regulation of lateral domain development has also been studied in cotton, where mosaic analysis has been used to characterize the activity of the *Okra* mutation (Dolan and Poethig, 1998b). This semi-dominant mutation leads to an exaggeration of lobing in the normally palmately lobed leaf, by increasing the length of lobes while at the same time suppressing growth between them. In sectorial chimeras, in which somatic chromosome loss has produced sectors in which *Okra* is missing in L1, L2 and L3, there was a close correspondence between with between the presence of *Okra* and abnormal lobing, suggesting that the *Okra* gene product does not diffuse significantly in a lateral dimension. On the other hand, in mericlinal chimeras, in which the fundamental layers of the lamina consist of a mixture of *Okra* and anueploid cell types, the phenotypic boundaries appeared more diffuse, suggesting the existence of non-autonomous mechanisms that reconcile conflicting growth patterns in different layers. Mericlinal chimeras also illustrate the distinct activity of the *Okra* gene product in each cell layer. On the basis of this analysis, the authors concluded that the growth of the lamina was most strongly influenced by the genotypes of the subepidermal and, to a lesser extent, epidermal tissues. By contrast, the genotype of the L3 layer, which typically contributes to the midvein, had little effect on the extent of lamina growth but did influence the length of the lobe.

7.5 Patterns of cell division associated with lamina development

7.5.1 The rise and fall of the marginal meristem

With the previous discussion focusing on establishment of the basic developmental axes of the leaf, it remains to be seen how basic growth processes are regulated to produce the mature leaf form. Of particular interest are underlying mechanisms that are capable of producing a lamina whose width and length are many times its thickness. Such a pronounced asymmetry has no doubt inspired

the formulation of models that would provide a simple explanation for such polarized growth. One long-standing explanation supposes the lamina derives from a 'marginal meristem', a hypothetical tissue consisting of initial-like cells arrayed along the leaf margin that would give rise to clones of cells that would extend inward from the leaf margin (Avery, 1933). The model is attractive in that the ultimate shape of the lamina would be determined through regulation of the mitotic activity of a spatially defined group of initials. While this model appeals by drawing a parallel with the apical development of the stem, it has failed to find support from a number of detailed analyses of cell division, which show marginal cells divide neither more frequently than internally positioned cells nor in a planes that would lead to preferred growth in a lateral dimension (Maksymowych and Erickson, 1960; reviewed in Poethig, 1984). More recently, a relatively uniform distribution of mitotic activity across the developing lamina was demonstrated quite clearly by a study in which a β -glucuronidase (GUS) reporter gene, driven by the promoter of a *cdc* gene, was used as a marker for cell division (Donnelly *et al.*, 1999). The existence of marginal meristems has also been questioned on the basis of clonal analysis studies, in which the distribution of cell division across the developing lamina appears to be relatively uniform (Poethig and Sussex, 1985; Poethig, 1987; Dolan and Poethig, 1998a). The preponderance of marginal clonal sectors that tend to run parallel to the leaf margin would suggest that marginal cells actually contribute more to the periphery of the lamina than to its lateral dimension.

A more strongly supported alternative that would explain the lateral growth of the lamina envisages a tightly integrated regulation of mitotic activity across the lamina, with no well-defined foci of cell division. Patterns of variegation seen in the leaves of periclinal chimeras are consistent with this view, showing that a consistent leaf shape can be obtained despite highly variable contributions from L1, L2 and L3 layers (Tilney-Basset, 1986; see also figure 7.5). Moreover, altered patterns of cell division may have little effect on the lamina shape. The

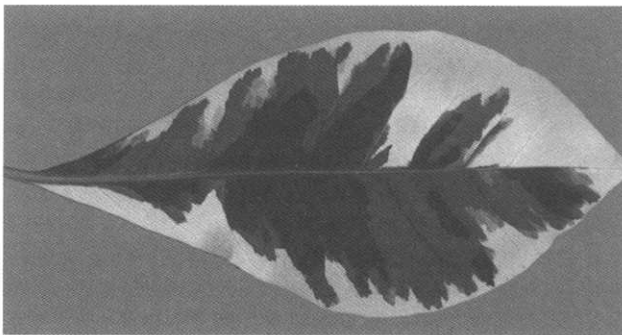


Figure 7.5 Periclinal chimera of a *Ficus* leaf, illustrating the variable contributions of the L1, L2 and L3 layers. (B. Veit, unpublished data.)

maize mutant *tangled* provides a case in point, in which severe perturbations to the regular patterns of transverse divisions in the leaf has relatively little effect on leaf shape (Smith *et al.*, 1996; see also figure 7.6). Similarly, increases in the frequency of cell division caused by the misexpression of the cell cycle regulatory gene, *cdc2* kinase, are also notable for their lack of effect on leaf form (Hemerly *et al.*, 1995). Taken together, these results are consistent with some form of global patterning, by which local variability in cell division patterns is assessed and somehow compensated to give consistent shapes and forms (Meyerowitz, 1996). Such a mechanism would monitor and regulate the extent of cell division throughout the leaf primordium, rather than acting on a set of hypothetical initials arrayed in a narrow strip along the margin of the leaf primordium. This more diffuse pattern of regulated cell division, which leads to increases in the area of the lamina, is sometimes held up as an example of intercalary meristem. Although such a meristem resists a simple spatial definition, it has utility in directing attention to temporally and spatially regulated patterns of cell division that ultimately yield predictable forms.

7.5.2 Intercalary meristems

Studies of the frequency and distribution of cell division along the proximal/distal axis present a more complex picture. Clonal analyses in tobacco, cotton and maize (discussed in section 7.4.2) provide no evidence for a SAM-like activity at the apex of the early leaf primordium, as was suggested by earlier workers (Avery, 1933). Analysis of the size, frequency and distribution of x-ray

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Figure 7.6 A comparison of epidermal cell division between the leaves of normal and *tangled* plants, illustrating the irregular pattern of division in the mutant. Arrowheads point to shallow indentations indicative of recent transverse (t) and longitudinal (l) divisions. Scale bars = 30 μm . (Smith *et al.*, 1996, *Development*, **122**, reproduced with permission of the Company of Biologists Ltd.)

induced sectors in tobacco suggest that, during early phases of leaf development, divisions are distributed relatively uniformly along the proximal/distal axis, an observation in accord with measurements of mitotic indices (Poethig, 1984). However, as the leaf begins to mature in a basipetal direction, there is a parallel decrease in the frequency of cell division, such that the basal regions of the leaf continue to divide longest. In maize, a similar picture emerges, with an initially relatively uniform distribution of cell division along the proximal/distal axis of the primordium shifting basipetally as the leaf matures. In neither maize or tobacco did large discrete sectors emanate from the leaf tip, arguing against cells along the proximal/distal axis being laid down by a SAM-like activity located at the primordium tip.

This pattern of localized cell division in the basal region of the leaf has sometimes been referred to as a form of intercalary meristem, especially in monocotyledonous plants, where its clearly defined position along the length of the strap-like leaf is especially apparent. While it is unclear what mechanisms might limit this persistent mitotic activity to the leaf base, it is possible that this pattern could merely reflect the normal basipetal pattern of maturation, with differentiated tissue being inherently less mitotically active. Alternatively, the position of the intercalary meristem might be actively specified by mechanisms that directly regulate cell division. In grass leaves, an intercalary meristem that becomes evident at the base of the primordium early in its development divides into a proximal and more distal meristem that give rise to the sheath and blade portions of the leaf, respectively (Dale, 1988). During the period that the intercalary meristem is active, the polarity of cell division changes, with narrow sectors in the distal part of the blade indicating a predominantly longitudinal bias, while broader sectors at the base indicate a less biased plane of division (Poethig, 1984).

The expansion of cells is another significant parameter associated with the growth of leaves, the role of which has been approached through both quantitative and genetic analyses. As seen for cell division and maturation, there is a basipetal pattern of expansion, which accounts for the majority of growth during later stages of leaf development. Insight into the regulation of expansion has been gained through the analysis of mutants that perturb expansion in a polar manner (Tsuge *et al.*, 1996). The *rotundifolia* (*rot*) mutant of *Arabidopsis* produces abnormally broad leaves as a result of decreased cell expansion along the proximal/distal axis of the leaf, while expansion across the width of the leaf remains normal. This abnormal expansion can be attributed to the disruption of a cytochrome P450 gene (Kim *et al.*, 1998). Overexpressing the normal gene leads to the formation of abnormally long leaves (Kim *et al.*, 1999), supporting the role of *ROT* in mediating the longitudinal expansion of cells. These authors suggest that the activity of the *ROT* gene might be mediated by brassinosteroids, given the similar effects of this class of phytohormone on elongation of stems and leaves, and the structural similarity of the *ROT* gene to genes involved in

brassinosteroid synthesis. By contrast, mutants to the *ANGUSTIFOLIA* (*AN*) gene have abnormally long leaves. Within these leaves, cells appear narrower across the width of the leaf, suggesting that the gene is required for normal expansion along this axis. Both the *rot* and *an* mutants can be contrasted with the previously described Okra mutants, in which no consistent changes in cell shape could be correlated with changes in leaf shape.

7.6 Regulation of determinacy

7.6.1 Acquisition of determinacy in leaves

Much of the previous discussion has focused on simple leaves whose pattern of development is readily distinguished from that of the SAM. As noted in section 7.5.2, many of these differences can be described in terms of meristematic growth patterns. The leaf owes its planar organization to the establishment of a dorsiventral axis and the concomitant formation of intercalary meristems responsible for the lamina formation. Just as significant, however, are differences that account for the determinate nature of the leaf, which can in part be attributed to the generally limited capacity for apical growth of the leaf. While the emergence of the leaf primordium fixes its position on the flank of the apical dome and establishes a proximal/distal growth axis, several lines of evidence (reviewed in Steeves and Sussex, 1989) suggest that the determinate nature of the leaf becomes fixed later in its development. In ferns, the behaviour of cultured explants of leaf primordia suggest that their normally determinate pattern of growth is acquired after emergence, with primordia gradually losing the capacity to convert to indeterminate, shoot-like structures up to approximately the P8 stage. These experiments suggest that the leaf acquires its determinate character gradually, in a manner that requires communication with the shoot apex.

In angiosperms, the acquisition of determinacy seems to take place much earlier, as primordia explants of sunflower and tobacco as young as P2 consistently develop as determinate leaves (as reviewed by Steeves and Sussex, 1989). These authors also point to studies in *Phaseolus*, in which a shoot-like structure arose from the P - 1 site, but only if the remainder of the shoot apex was removed. Similarly, in *Nuphar*, shoots occasionally emerged from P1, P - 1 and P - 2 when these sites were isolated from the apex by tangential incisions, suggesting that in angiosperms, like ferns, the determinate character of the leaf is acquired via some sort of communication with the shoot apex.

7.6.2 Compound leaf development and intermediate states of determinacy

In the case of compound leaves (Goliber *et al.*, 1999), the distinction between leaf and stem becomes less clear and points to affinities that have intrigued

generations of plant biologists and philosophers (Arber, 1950). Like the stem, the compound leaf produces laminar structures that for all intents and purposes resemble leaves. These leaflets, or pinnae, may be borne at periodic intervals along a rachis as in pinnately compound leaves, or alternatively, spread from a restricted region at the end of a petiole as in palmately compound leaves (Hickey, 1973). While pinnae can be likened in many respects to forms seen in simple leaves, they are distinguished by the absence of axillary meristems. By contrast, the primary axis on which leaflets are borne generally subtends an axillary bud.

The compound leaves, as a whole, may also be compared to the shoot in terms of its less determinate pattern of growth. In some species, this indeterminacy is manifest in the pattern of apical development. In contrast to the typical pattern of leaf development, in which apical growth becomes limited at very early stages of primordial development, a number of species with pinnately compound leaves have been described, in which apical growth appears less determinate. Within the family *Meliaceae* exist several tropical genres, including *Guarea* (Steingraeber and Fisher, 1986) and *Chisocheton* (Fisher and Rutishauser, 1990), in which the main axis of the pinnately compound leaf may initiate leaflets over a period of several years. This indeterminacy, together with a pattern of secondary thickening seen along the rachis, led early workers to describe the *Guarea* leaf as a stem. Subsequent workers have favoured a leaf identity on the basis of the main axis of the leaf subtending an axillary bud and the existence of a heteroblastic series in which simple determinate juvenile leaves precede the formation of a compound form. In *Chisocheton*, the stem-like character of the leaf is further suggested by the emergence of buds on the adaxial surface of the leaf rachis between pairs of pinnae (Fisher and Rutishauser, 1990).

The indeterminacy of apical growth seen in the compound leaves in species such as *Guarea* has prompted the question of whether SAM-like structures might be present. Many studies have offered images generated by scanning electron microscopy (SEM) or with sectioned material, in which the acropetal sequence of leaflet initiation along the rachis axis bears a striking resemblance to patterns that might be observed for the initiation of leaves from stems of species with a distichous or opposite phyllotaxy (e.g. Rutishauser, 1995). Like leaf primordia, leaflet primordia emerge at periodic intervals in a subapical position and soon develop a flattened bilateral symmetry that closely parallels that of simple-leaved forms. It is intriguing that in many plant groups, the plane of bilateral asymmetry of the leaflet is initially perpendicular to the main axis of the leaf, and thus closely mirrors the similar orientation of leaf primordia with respect to the main shoot axis (Troll, 1937). On this basis, it might be hypothesized that the tip of the developing leaf functions much like the SAM in providing cues for the establishment of dorsiventrality in lateral organs.

Despite evidence for prolonged apical growth of the primordial leaf axis and the parallels that can be drawn with organogenic functions of the shoot apex, the case for a SAM-like structure remains equivocal. Although for some

compound forms, apical growth appears to lead the acropetal pattern of leaflet initiation, it is unclear whether initiation is mediated by a SAM-like activity. Some workers have also drawn attention to a tunica/corpus-like apical region of leaves that show indeterminate growth (Lacroix and Sattler, 1994). However, this organization may largely reflect the predominantly anticlinal division patterns usually associated with dermal cell lineages. Direct measurements of mitotic activity that might point to a population of slowly dividing cells analogous to the central zone are lacking for these compound leaf types. Similarly, information on lineage relationships among cells along the proximal/distal axis of the leaf, which might reveal the presence of long-lived initials, is also lacking. The patterns of leaflet emergence cast further doubts on whether their initiation can be linked to a SAM-like activity. In contrast to the acropetal sequence of leaf emergence from the SAM, leaflets may emerge in a basipetal or divergent sequence along the rachis (Troll, 1937), suggesting that leaflet initiation is not tightly coupled to apical growth. Examples of compound forms from related genera that differ in the direction of leaflet initiation suggest an intriguing plasticity for this character (Sugiyama and Hara, 1988).

To account for these complex patterns of leaflet initiation, an alternative model of lamina growth and leaflet initiation has been proposed (Hagemann, 1970), which supposes that leaflet primordia originate via a process termed 'meristem fractionation'. By this process, marginal meristematic regions of the young leaf primordium become discontinuous, leading to alternating regions of meristematic and non-meristematic tissues along the leaf margin, which may lead to foci of lamina outgrowth or initiation sites for leaflet development. Studies of compound leaf development in ash have been interpreted to support a process of fractionation (Merrill, 1986a,b and c). A continuous line of meristematic tissue along the lateral axes of the young leaf primordium, recognized in terms of dense staining and increased mitotic activity, becomes discontinuous through the development of intervening, more slowly dividing vacuolated regions. More recently, the term *blastozone* has been proposed, which like the term marginal meristem describes localized regions of growth associated with organogenesis, but differs in not being tightly linked to differences in cytological characters or idiosyncratic patterns of cell division per se (Hagemann and Gleissberg, 1996). This change in terminology addresses many instances in which polar patterns of growth are not associated with changes in the frequency or orientation of cell division, and recognizes a widely held view that such growth is not a simple outcome of division behaviour.

Although no detailed explanation is offered for fractionation of the marginal meristem, its origin is discussed in relation to the SAM. In this model, a *blastozone* comprising the SAM is fractionated during leaf initiation such that a continuous marginal meristem forms along the lateral margins of the leaf as the primordium diverges from the shoot apex. By this model, marginal meristems of the leaf primordium are a by-product of the fractionation process, forming

at the fission boundary between the two structures. The authors argue that, by this mechanism, the leaf primordium would have a defined dorsiventral axis of symmetry from its inception, with growth axes of the leaf also defined by a fan-shaped fission boundary between the leaf and shoot. While the model provides an explanation for the location of and polarity of marginally directed growth centres, it is difficult to reconcile with previously discussed surgical and genetic studies that suggest that the dorsiventral axis of the leaf is determined in a progressive manner after it is initiated.

7.6.3 Promotion of indeterminacy by *KN1*-like genes

Despite these apparent conflicts, recent molecular genetic analyses have revealed patterns of gene regulation that link indeterminate patterns of growth of the leaf and SAM. As discussed in section 7.4.1, *KN1*-like genes are consistently expressed in the SAM, but are downregulated in the anlagen in the simple leaf primordia of maize and *Arabidopsis*. When overexpressed, however, these genes confer less determinate patterns of leaf development, which include lobing and the development of shoots that originate from the surface of the lamina (Matsuoka *et al.*, 1993; Sinha *et al.*, 1993; Lincoln *et al.*, 1994; Chuck *et al.*, 1996; Tamaoki *et al.*, 1997; see also figure 7.7).

The association between the expression of *KN1*-like genes and indeterminate patterns of leaf development is further strengthened by studies in tomato, in which overexpression leads to striking increases in the degree of ramification of the normally compound leaf. For example, overexpression of the maize *KN1* gene results in the formation of super-compound leaves, each bearing up to 2000 leaflets (Hareven *et al.*, 1996; see also figure 7.8). Subsequent studies

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Figure 7.7 Formation of ectopic inflorescence meristem on the adaxial surface of *Arabidopsis*. Arrow points to an ectopic stipule. Abbreviation: cl, cauline leaf (Internal scale bar = 30 µm). (Reprinted from *Plant Cell*, 8, Chuck *et al.*, pp. 1277-1284, 1996, with permission. Copyright held by the American Society of Plant Biology.)

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Figure 7.8 Effect of overexpressing the *KN1* gene of maize in tomato: A) normal compound leaf; B) super compound leaf from overexpressing plant. Abbreviations: TL, terminal leaflet; LT, lateral leaflet; F, floret; P, petiole; R, rachis. (Reprinted from *Cell*, **84**(5), Hareven *et al.*, The Making of a Compound Leaf. . . , pp. 737-739, 1996, with permission from Elsevier Science.)

have revealed that the normal compound nature of the tomato leaf is also linked to the persistent expression of the endogenous *KN1*-like genes, *TKn1* and *TKn2*, in leaf primordia (Chen *et al.*, 1997; Parnis, 1997). Although highly expressed in the SAM, these genes differ from their *Arabidopsis* and maize counterparts in not being downregulated in the P0 anlagen of leaf primordia. Instead, expression persists in the developing leaf, especially in regions corresponding to provascular tissue and developing leaflet primordia. However, despite the normal expression of *KN1*-like genes in the developing leaf, augmentation of this expression in the leaf leads to further increases in the degree of ramification, as well as the formation of ectopic shoots on the lamina and in the pseudoaxils of leaflets (Janssen *et al.*, 1998). Thus, it seems likely that the regulation of *KN1*-like genes plays a key role in limiting the potential for indeterminate growth.

7.6.4 Context-dependent limitations on indeterminacy

It is also clear from the preceding discussion that the consequences of expressing *KN1*-like genes are limited by context. Apart from previously discussed subtle changes in plastochron ratio, the effect of overexpression of *KN1*-like genes on the SAM appears minor. Within the leaf, the outcome differs depending on the species. As described above, overexpression of *KN1*-like genes in tomato leads to indeterminate growth of the leaf and associated increases in the degree of ramification. However, overexpression of essentially identical constructs in *Arabidopsis* or tobacco leads only to increases in lobing, and the sporadic formation of ectopic shoots. Therefore, it would seem that simple leaf shape does not solely reflect the absence of *KN1*-like gene products in the developing primordium but also indicates other limiting factors. Tomato would appear to have an unusual programme of leaf development that responds to *KN1*-like genes by promoting indeterminate branching.

Leaf shape mutants of tomato afford additional examples of how the effects of *KN1*-like genes are limited by genetic context (figure 7.9). One of these, conditioned by the semi-dominant *Lancelolate* mutation, converts the compound leaf to a simple entire form (Dengler, 1984). Interestingly, this mutant responds to the overexpression of *KN1* in a manner very similar to *Arabidopsis* and tobacco, by conversion of the leaf to a highly lobed form without increases in the degree of ramification (Hareven *et al.*, 1996). Similarly, the effect of overexpressing *KN1* in the *potato leaf* mutant, in which leaves are compound but with entire leaflets, also seems to be more limited than in wt. Leaflet number in the mutant is unchanged, but the form is modified, with an increase in petiole length and ramification of terminal portions of the midveins.

The analysis of tomato mutants also suggests a relationship between separate leaflets and highly lobed leaves. In the *trifoliolate* mutant, in which a ternate

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Figure 7.9 Tomato mutants that limit the effects of *KN1* overexpression. A) wt; B) *Lancelolate*; C) *Lancelolate* + *KN1* overexpression; D) *potato leaf* versus *potato leaf* + *KN1* overexpression; E) and F) *trifoliolate* versus *trifoliolate* + *KN1* overexpression. (Reprinted from *Cell*, **84**(5), Hareven *et al.*, The Making of a Compound Leaf. . . pp. 737-739, 1996, with permission from Elsevier Science.)

leaf is normally seen, *KN1* overexpression appears to enhance lobing such that each leaflet also assumes a ternate form. This conversion suggests a relationship between compound and highly lobed leaf forms seen among members of various plant groups, since an extreme lobing, which dissects the lamina to the rachis, would convert the lobe to a separate leaflet. In a number of plant groups, a continuum from lobed to fully dissected forms can be observed. For example, in the *Ranunculaceae*, heteroblastic series exist in which palmately compound leaves follow on from palmately lobed forms. A similar continuum can also be observed among closely related species, such as within the genus *Acer* (figure 7.10).

7.6.5 Mechanisms by which *KN1*-like genes promote indeterminacy

A molecular explanation for how the leaf might acquire its determinate character has begun to emerge through analysis of the functions of *KN1*-like genes in the SAM. As discussed in section 7.6.3, the expression of genes in this class is linked to indeterminate patterns of growth in both the SAM and, in some cases, the leaf. Insights into how these patterns of growth might be effected have begun to emerge with the identification of downstream targets of *KN1*-like genes. Earlier work had shown that in tobacco, ectopic expression of *KN1*-like genes leads to large decreases in the levels of bioactive gibberellin (Tamaoki *et al.*, 1997), an effect that appears to be mediated by decreased activity of one or more gibberellic acid (GA) biosynthesis genes (Tanaka-Ueguchi *et al.*, 1998). Subsequently, it was shown that one of these genes, GA 20-oxidase (*Ntc12*) is a direct target of repression by *NTH15*, a *KN1*-like protein (Sakamoto *et al.*, 2001). Like other *KN1*-like genes, *NTH15* shows a typical pattern of expression, with downregulation in the SAM in regions corresponding to incipient leaf primordia. This pattern of expression is consistent with decreases in GA levels seen upon overexpression, and would suggest that normal SAM function may depend on low levels of GA in constituent tissues, while normal patterns of leaf development depend on its de-repression. The amelioration of aberrant leaf phenotypes associated with overexpression of *KN1*-like genes by exogenously supplied GA, or by mutants that result in constitutive activation of the GA pathway lend weight to this model (M. Tsiantis, unpublished results).

7.6.6 Homeotic transformations in the compound leaf of pea

Significant insights into compound leaf development have also been gained by studies of the oldest genetic system, the common garden pea. The plant normally produces pinnately compound leaves, with an acropetal sequence of development yielding a pair of foliar stipules, a pair of proximal leaflets, two pairs of tendrils and a single terminal tendril. Surgical and explant studies suggest that the different domains of the leaf are also determined in an acropetal

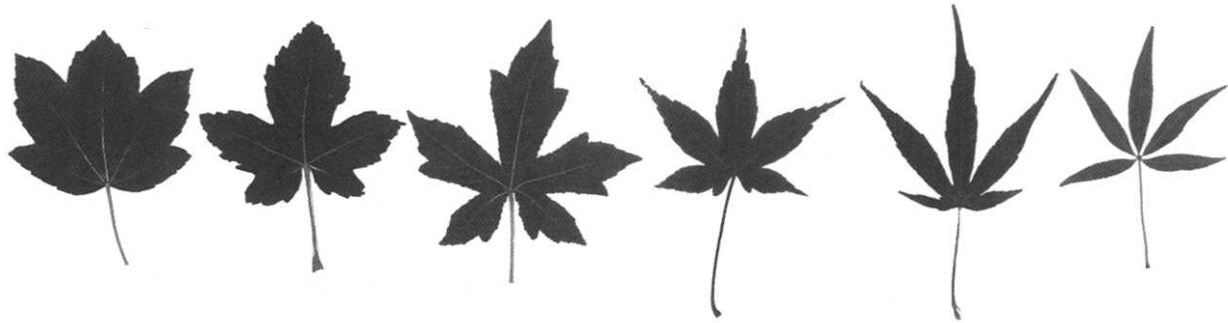


Figure 7.10 A comparison of leaf forms of different species of *Acer* showing the progression from palmately lobed to palmately compound forms (B. Veit, unpublished data.)

sequence (Gould *et al.*, 1994). Stipules are determined first, with lateral and terminal domains of the distal part of the leaf still undetermined in primordia less than 70 μm in length (Sachs, 1969).

The basis for this stereotyped pattern of development has been approached through analysis of leaf mutants (Marx, 1987; see also figure 7.11). The *tendriless* mutant (*tl*) leads to the replacement of tendrils with leaflets, while the *afila* (*af*) mutant has the opposite effect, transforming leaflets into branched tendrils. The homeotic character of these transformations is suggested by the constancy in organ number and position along the leaf rachis, as well as by anatomical studies that demonstrate a clear correspondence between organs formed at ectopic positions with their normally positioned counterparts. Based on these transformations, Marx proposed that the morphologically distinct regions of the pea leaf could be viewed as 'compartments' which somehow become committed to distinct programmes of development.

Various models have been proposed to explain how regional patterns within the pea leaf are specified. A simple model (Young, 1983) focuses on the acropetal pattern of leaf development, supposing that the identity of organs along the developing leaf axis is determined by the size of the primordia from which they initiate. Thus, a relatively large primordium born on the stem axis would develop as the rachis of the leaf, while progressively smaller primordia formed along the rachis would develop into leaflets or tendrils, depending on their size. Mutations

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Figure 7.11 Morphology of wild-type and mutant pea leaves. A: wt leaf composed of a pair of basal stipules, a pair of leaflets and distal tendrils. B: *uni* mutant in which distal regions have been reduced to a unifoliate form. C: *tl* in which tendrils are replaced by leaflets. D: *af* mutant leaf in which leaflets have been replaced with tendrils. E: *af tl* double mutant in which all positions on the blade region are occupied by branching rachides terminating with small leaflets. F: *coch* mutant showing replacement of stipules with compound leaf-like structures. (Reprinted from *The Plant Cell*, **12**, Gourlay *et al.*, pp. 1279-1294, 2000, with permission. Copyright held by the American Society of Plant Biology.)

in homeotic genes would perturb the normal pattern of pinnae development by either altering size thresholds that would normally distinguish pinnae identities or altering the size of primordia. Thus, for example, mutations in *TL* might convert tendrils into leaflets by increasing the size of distal primordia, or by decreasing the size threshold required for leaflet formation. While attractive in terms of its simplicity, the model is not supported by anatomical studies, which fail to show the expected gradient in primordia size in normal leaves or changes in primordia size among the *tl* and *af* mutants.

An alternative model for the specification of pinnae identity has been proposed (Marx, 1987) which attributes regional differences to the domain-specific activity of homeotic genes. By this model, *AF* would normally act specifically in proximal regions of the leaf to either repress a tendril programme or promote a leaflet programme. Conversely, *TL* could be hypothesized to act in terminal regions of the leaf, to either suppress a leaflet programme or activate a tendril programme. This model, in its simplest form, has been criticized as it fails to explain intermediate double mutants of *tl* and *af*, in which tendril-like rachides terminated with small leaflets occur at all positions along the leaf rachis. To account for these structures, it has been proposed that the double mutant should be viewed as a ground state for the compound architecture of the pea leaf, with *TL* and *AF* acting to promote distinct pinnae identities. A model proposed by Lu *et al.* (1996) supposes that regional differences in pinnae identities are determined by a heterochronic mechanism, in which the process of lamina initiation is promoted by *AF*, thereby precluding branching and tendril formation, while *TL* would normally counteract this activity and also suppress branching. In *tl*, lamina initiation in terminal regions would be de-repressed, leading to the conversion of distal pinnae into leaflets. By contrast, loss of *AF* activity would lead to an overall lower level of lamina-promoting activity, leading to the alternative pattern of tendril formation in the proximal region of the leaf. Loss of both *AF* and *TL* would lead to both de-repressed branching and delayed lamina formation to yield the highly ramified leaflet terminated structures seen in the *tl af* double mutants.

While it is clear that morphological, anatomical and developmental analyses have provided the essential background upon which the development of the pea leaf can be modelled, initial molecular genetic studies have offered some unexpected insights. The loss-of-function mutant phenotype of the *UNIFOLIATA* (*UNI*) gene, which reduces the compound leaf to a simple lamina, suggests *UNI* plays a key role in the determination of the compound form (Hofer *et al.*, 1997). Unlike previously described leaf mutants, *uni* has an altered pattern of floral development, in which a profusion of sepal- and pistil-like organs replace petals and stamens. This pattern suggested a possible correspondence with several cloned genes from model systems that exhibit similar loss-of-function phenotypes. Mapping and cloning experiments have confirmed this hypothesis, demonstrating that *UNI* is orthologous to *FLO* of *Antirrhinum* or

LFY of *Arabidopsis*, both of which have been shown to play a key role in the floral meristem determination. *In situ* localization studies have shown that like *FLO* and *LFY*, *UNI* is strongly expressed in the petal and stamen primordia of developing flowers. In addition, expression of *UNI* is also observed in developing leaf primordia, especially in blastozone regions corresponding to developing pinnae. More recent studies have extended these observations, linking more persistent patterns of *UNI* expression with the more indeterminate patterns of branching in *tl* and *af_{tl}* (Gourlay *et al.*, 2000). This correlation is also seen in the *cochleata* mutant, where the conversion of simple to compound stipules is linked to the ectopic expression of *UNI*. The connection between the expression of *LFY*-like genes and less determinate patterns of leaf development is also indicated by the mutant phenotype of the tomato orthologue of *LFY*, *falsiflora*. Like *uni*, the loss-of-function *falsiflora* mutant results in altered floral development as well as leaves that have fewer and smaller lateral leaflets. By contrast, there are no clear examples where expression of *LFY*-like genes has increased the complexity of leaves in simple-leaved species, such as *Arabidopsis*. While artificial overexpression of *LFY* in *Arabidopsis* leads to precocious flowering, no obvious changes in the pattern of leaf development are observed. It would therefore seem that the capacity of *LFY*-like genes to promote less determinate patterns of leaf development might be limited in simple-leaved species.

Current models for the function of *LFY*-like genes offer no obvious explanation of how they might cause less determinate patterns of leaf development. In the context of floral development, such genes appear to promote the conversion of the vegetative meristem to a floral state, as well as to activate expression of specific floral organ identity genes belonging to the MADS-box family of transcription factors. It is perhaps surprising that a *LFY*-like gene would be involved in promoting the more determinate fate of the floral meristem, while at the same time promoting a less determinate pattern of growth in the leaf. One common theme that could conceivably link the activity of *LFY* in both contexts might be the activation of a common set of target genes. In the context of the SAM, these would promote floral meristem identity, while in the context of the leaf, these same genes would somehow promote less determinate development along the leaf rachis.

While the molecular nature of these contextual differences is unclear, *KN1*-like genes, as well as others with contrasting patterns of SAM versus leaf expression, could be viewed as potential players. Studies of *KN1*-like genes in pea suggest that the less determinate growth of its leaf is unlike that of tomato, in that *KN1*-like genes are downregulated in leaf primordia as they are in simple-leaved species. Thus, it would appear that the pea leaf provides another example where the mere presence or absence of *KN1*-like genes in the leaf primordium does not entirely account for simple versus compound leaf architecture. An alternative hypothesis that might link the leaf and floral-related functions of *LFY* would suppose that the targets of *LFY* regulation differ between the SAM

and leaf primordium, and that its involvement in both processes grew out of the ability of *LFY* to respond to a common signal, such as GA (Blazquez and Weigel, 2000). Tests to distinguish these models will no doubt focus on the nature of *LFY* targets as defined through molecular and genetic strategies.

7.7 Morphology and mutants: shadows of forgotten ancestors?

At times, the distinction between leaf and shoot seems only too clear. As foliage peels away from branches in the crisp autumn light, it would be natural to view the two structures as distinct, incontrovertible types. In the light of fossil evidence, however, it seems that the leaf was in some way derived from stem-like progenitors, leaving us to ponder how. As mechanisms responsible for their distinct patterns of growth and development are revealed in molecular terms, we can begin to consider the questions set out at the beginning of the chapter: What is the leaf, and what is its relationship to the stem? What are the programmes of development that underpin the essential nature of the leaf, and to what extent do they overlap or differ with those of the stem?

7.7.1 'But what is the leaf': a morphological perspective

Our current concept of the leaf and its relationship to the stem is largely based on perspectives offered by comparative morphology. A set of criteria that describe the archetypal leaf and stem can be obtained through careful consideration of their ontogeny and final form. The leaf emerges at predictable positions on the flank of the stem and is typically associated with a meristem that forms in its axil. Its dorsiventral asymmetry can be contrasted with the radial symmetry of the stem. As the leaf develops, waves of cell division and maturation sweep across the primordium and vanish. By contrast, the SAM of the stem persists, essentially propelled ahead by a standing wave of subapical cell division and expansion.

While morphology focuses archetypal forms, it gives due attention to exceptions, thus providing a clear impression of the degree to which certain character states are linked. Thus, we have seen how a graded series of intermediate forms suggests a close relationship between pinnately and palmately compound leaf forms. For some species, leaves may display stem-like patterns of indeterminate growth, while simultaneously expressing more leaf-like traits. Conversely, in species with a sympodial architecture, branches may take on a determinate pattern of growth, but still retain other hallmarks of the stem, such as radial symmetry. Similarly, through distichous patterns of leaf initiation coupled with the absence of axillary buds, short shoots may take on the appearance of dorsiventral compound leaves (e.g. *Taxodium*). In other groups (e.g. *Ruscus*), the dorsiventral character of the stem and its associated vasculature closely approximates a leaf-like form (Arber, 1950). In their extreme form, such exceptions have been

held up as examples of 'fuzzy morphology', as they would appear to erode some of the fundamental criteria upon which morphological interpretation relies (Rutishauser, 1999).

From a mechanistic perspective, it could be argued that such 'fuzzy' exceptions may have exactly the opposite effect in terms of clarifying the degree to which specific patterns of development are coupled. The coincidence or independence of certain characters, be they molecular or morphological, provide valuable clues as to the nature of the underlying developmental mechanisms. To the extent that some sets of characters are always linked, it becomes plausible that they might be described in terms of a 'programme' of development. By contrast, independence between character sets seen across a number of taxonomic groups would suggest that characters are determined by more than one programme, or could be viewed as being determined by subprogrammes. Thus, morphological comparisons are valuable in having the potential to reveal the outlines of discrete developmental programmes that have been exploited by evolution to obtain a variety of leaf architectures. They also circumscribe programmes that would be most amenable to genetic analyses aimed at describing developmental processes in mechanistic terms.

7.7.2 *Genetic comparisons of the stem and leaf*

In earlier discussion, it was seen how model systems, to the extent they are amenable to genetic analysis, can provide some initial insight into the relationships between characters that distinguish the archetypal stem and leaf. Genetic analyses have illuminated the nature of differences in the determinacy of the stem and leaf. *KN1*-like genes appear to play a key role in maintaining indeterminate patterns of growth in the SAM, and in some cases, the leaf. Though highly expressed in the SAM, *KN1*-like genes were seen to be downregulated during the inception of the leaf primordia in maize and *Arabidopsis*, leading to the suggestion that leaf founder cell identity could be defined in these relatively simple terms. However, doubts concerning this simple model arose with the observation that *KN1*-like genes were expressed in tomato leaf primordia. The inspiration for this significant revision, found in a system with a 'variant' leaf architecture, emphasizes the need to carefully consider modes of development in non-model systems. Progress in this area will no doubt be aided by the generally high degree of conservation of developmental mechanisms among plants, providing a quorum of common players to highlight the presence of novel elements responsible for non-canonical patterns of development. Furthermore, advances in transformation technology and the development of robust methods to precisely enhance or inhibit gene expression will encourage genetic approaches in previously recalcitrant systems.

KN1-like genes also have relevance to the establishment of the proximal/distal axis, and possibly the dorsiventrality of the leaf. Ectopic expression of

KN1-like genes in developing leaves of maize results in a distal shift of proximal associated domains of the leaf. However, given that these genes are not normally expressed in the leaf, the relevance of their expression in the SAM to leaf development is unclear. While certain *KN1*-like genes, such as *KN1* itself, are expressed throughout the SAM except in leaf founder cells, others are expressed in more restricted patterns. The expression of *RS1* and *GN*, for example, is restricted to the base of the pre-emergent leaf. It therefore seems possible that certain members of the *KN1*-like gene family may function to establish boundaries within the SAM that function to pattern the pre-emergent leaf. Ectopic expression of such genes in the post-emergent primordia would lead to the formation of anomalous boundaries, thus distorting the ensuing development of the leaf. The supposed role of *KN1*-like genes in establishing such boundaries finds close parallels on both theoretical grounds (Meinhardt and Gierer, 2000), as well as in *Drosophila* development in which homeodomain genes play a central role. Analysis of loss-of-function mutants and transgenic plants, in which the patterned expression of *KN1*-like genes in the SAM is perturbed, should offer insight into whether *KN1*-like genes have analogous functions in patterning the pre-emergent leaf.

As described in detail in chapter 2 of this volume, key players in mechanisms that promote and maintain the growth associated with the SAM are beginning to emerge from the wings. Some of these, including previously discussed *KN1*-like genes, *CLAVATA 1*, 2 and 3, and *WUSCHEL*, appear essential for the establishment and/or maintenance of the SAM. The question could then be posed, 'What roles might genes of this sort have in the developing leaf?', if the leaf was in fact moulded from a system of naked branches as supposed by the telome theory. While expression of *KN1*-like genes is associated with the indeterminate pattern of growth seen in the compound leaf of tomato, such expression is not normally seen during leaf development, even during early stages of primordia formation. Similarly, there have been no instances in which *CLAVATA* or *WUSCHEL* like genes are expressed during leaf development. It could be argued that such genes, which serve to maintain a population of undetermined cells in the SAM, would be superfluous in a telome-derived leaf, given its more determinate pattern of growth. Such an interpretation would be consistent with the prominent role of intercalary meristems in the growth of the leaf, in contrast to the terminally positioned activity of the SAM. If so, a key step in the derivation of the leaf via the telome would be a shift away from mechanisms that direct meristematic growth from the apex to those that operate in intercalary positions. One expectation from this line of reasoning might be that mechanisms that regulate intercalary forms of growth in the stem, such as in the internode, might be homologous to mechanisms that coordinate intercalary patterns of growth in the leaf. Support for such homology awaits the outcome of molecular genetic analyses focused on these growth patterns.

A second key question posed to the telome theory relates to the dorsiventral character of the leaf. The lamina is supposed to originate through the planation of a series of dichotomously branched stems, followed by a webbing process in which lamina tissue gradually fills in gaps between the branch systems. Recent progress towards explaining dorsiventral patterning of the leaf would suggest that a telome origin requires a fundamental change in how dorsiventrality is induced and expressed relative to adjacent branches. In the case of the leaf, the plane of dorsiventral asymmetry is perpendicular to the adjacent stem axis. By contrast, the webbing of a telome would derive from lamina elements whose plane of symmetry is parallel to the plane of adjacent stems. A more detailed understanding of mechanisms that effect dorsiventral patterning and development promises further insight into the relationship between the leaf and stem. Some insights have begun to emerge through the analysis of genetic factors, including the *PHAB*, *PHAN*, *RS2* and *YABBY* genes. The differentiation of adaxial versus abaxial cell types appears to be linked not only to programmes that yield specific tissue types, but also to the regulation of SAM activity. While the nature of these interactions is unclear, the likely function of these genes as transcriptional regulators provides an obvious line of inquiry. What are the functions of their targets, and how does the activity of these targets differ in the stem and leaf?

A third issue concerns the veins of the leaf. Can they be considered homologous to a flattened network of stems wearing the mantle of the lamina? If the primary veins reflect the evolutionary remnants of the leaf's telome origin, does their pattern of branching recall the primitive patterns of stem branching from which the telome was fashioned? What factors control the vascularization of the leaf, and to what extent do these also operate in the stem? In chapter 6 of this volume, evidence that builds upon the well established role of this plant growth regulator in inducing vascular development is presented for auxin playing a key role in the initiation of the leaf. These studies also highlight the distinct character of vascular development in the leaf versus stem, as illustrated by the distinct responses to auxin or its inhibitors, as well the prominent expression of *KN1*-like genes in the developing vasculature of the stem, but not the leaf. Recent progress towards characterizing key genes affecting auxin transport and responses, as well as genes directly involved in vascular development promises a deeper understanding of the relationship between the vascular systems of the stem and leaf.

The foregoing discussion should not be taken to undermine the validity of the telome theories for leaf development, but is instead intended to stimulate lines of inquiry that combine traditionally disparate perspectives. How can trends suggested by the fossil record and comparisons among extant groups be reconciled with the emerging understanding of mechanisms that regulate the growth and development of the plant? How have the ancestral mechanisms

that gave rise to simple fossil forms been modified to yield the diverse forms we see today? Does the hierarchical patterning of the leaf revealed by genetic approaches suggest simpler alternatives to the telome theory? Does the leaf represent the product of a fundamentally new form of meristematic growth, or is it instead a reshaped form that embodies a pre-existing complexity? The next century promises many exciting answers through the application of molecular genetic approaches to questions framed by comparative morphology.

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8 Regulation of cell division in plants

Walter Dewitte and James Murray

8.1 Introduction

In higher plant meristems, the mitotic cell cycle is essential to maintain a population of stem cells in order to compensate for loss of cells through organogenesis. Furthermore, the initiation of new organ primordia in the meristem is accompanied by a localised increase in cell division (Lyndon, 1998). The mitotic cell cycle is not a real cycle, but rather a process during which a single cell separates itself into two daughter cells. The cells themselves do not cycle. The expression 'cycle' refers to the duplication of the nuclear genetic material and its subsequent equal distribution between the daughter cells, resulting in two cells with identical genetic information. Intertwined with this cycle of the genetic material is a cellular growth cycle. The entire cell participates in these actions, and these events can only be realised successfully if they are executed in a highly coordinated manner, which implies an ordered series of molecular and cellular processes. During the last three decades, some of the key players have been characterised. This chapter surveys our current knowledge of the molecular mechanisms involved in the cell cycle and its control, and guides the reader to literature in this field. So far as the availability of data permits, molecular knowledge is linked to the events of the cell cycle. Furthermore, the cross-talk between cell cycle machinery and developmental organisation in meristems is explored.

8.2 The cell cycle machinery of plants—a 'symphonic' survey

8.2.1 *'The movements': events of cell division and their quality control*

The cell cycle of eukaryotes can be divided into four sequential phases, designated G1, S, G2 and M. DNA-replication (S-phase) and the segregation of the chromosomes (M-phase) are separated by two gaps. The G1 phase (first gap) separates mitosis from the next S-phase, and the G2 phase (second gap) is interposed between the S- and M-phases. Consequently, G2 is discriminated from G1 by the doubled DNA content of the cells. Cells are capable of arresting at the G1/S transition or the G2/M transition according to the plant model and the tissue, indicating the importance of these boundaries in the control of the cell cycle. Furthermore, these transitions have also been shown to act as primary 'checkpoints' for oxidative stress (Reichheld *et al.*, 1999).

Before the commitment to divide, integration of intracellular and extracellular signals that determine whether or not conditions are favourable for cell division must occur. This includes probing the nutrient status of the cell, its position within the tissue, stress conditions and its degree of differentiation. It is likely that the evaluation of this information is achieved by a complex set of interacting signalling pathways that transduce the information to the cell cycle machinery. Furthermore, it is likely that the integrity of DNA is monitored, and if necessary, repair occurs prior to the onset of DNA synthesis during S-phase. Molecular evidence for this mechanism in plants is now beginning to emerge (Britt, 1999; Deveaux *et al.*, 2000).

Following commitment, in G1, of the cell to cycle, the chromosomal DNA and the chromatin structure is replicated during the S-phase and the identical sister chromatids remain attached to each other at several sites. Chromosomal DNA replication starts at loci termed 'origins' and is initiated by complexes of MCM proteins, a conserved process among eukaryotes (Sabelli *et al.*, 1998). The replication itself is catalysed by the 'replication complex', which involves DNA polymerases, DNA helicase and other proteins.

After the duplication of the chromosomes, the cytoskeleton is completely reorganised early in the G2 phase. The interphase microtubule array, consisting of filaments of microtubules beneath the plasma membrane, is degraded. Following this, a new structure, unique to plant cells, appears. This structure, the preprophase band, is the result of the assembly of cortical microtubules in a ring-like organisation whose position exactly marks the future cleavage plane. Concomitant with the appearance of the preprophaseband, the nucleus is surrounded by a vast assembly of microtubules, preceding the construction of the mitotic spindle (for a review, see Vantard *et al.*, 2000). By analogy with animals and yeast, it can be expected that in plants the entry into the M-phase, during which the actual event of cell division occurs, is accompanied by an important additional quality check. Prior to the breakdown of the nuclear envelope, the cell verifies and requires that each chromosome has completely duplicated to avoid the production of daughter cells with an incomplete sets of chromosomes. The dependency between these cell cycle events is evidenced by the fact that incomplete chromosome duplication caused by chemical inhibition of a DNA polymerase prevents progression of cell division.

The actual cell division event in plants during M-phase is again a sequence of temporally well separated and defined events, each of them with their check-points. During prophase, the chromosomes condense and the mitotic spindle is initiated around the nucleus. The preprophase band disappears and the nuclear envelope is degraded, resulting in an open mitosis. The chromosomes align in the centre of the mitotic spindle during the metaphase, followed by the breakdown of the connections between the sister chromatids that indicates the beginning of the anaphase. During anaphase one set of chromatids is being pulled towards each pole of the mitotic spindle. When compared with animals, higher plants

have developed a different mechanism for the partitioning of the cytoplasm between the daughter cells. In somatic plant cells, cytokinesis is initiated in the centre and progresses to the parental cell wall. This process includes the *de novo* formation of the plasma membrane and cell wall. The phragmoplast, which is a dynamic cytoskeletal array, directs membrane vesicles to the centre of the division plane. These vesicles fuse and form a membrane compartment, the cell plate. During maturation, material for the new cell wall is secreted into the lumen of the cell plate (Staehelin and Hepler, 1996).

8.2.2 *Instruments of the orchestra and the style of conducting: cyclin-dependent kinase complexes*

8.2.2.1 *Cyclin-dependent kinases*

Cyclin dependent kinases (CDKs) are a conserved family of serine-threonine protein kinases in eukaryotes. Their function, together with antagonistic phosphatases, is to regulate different cellular processes, including the cell cycle, by reversible phosphorylation of proteins. In most model systems, their functionality requires complex formation with a regulatory subunit, termed a cyclin. The first members of the CDK family were identified in screens for cell division cycle mutants in yeast, resulting in the use of the abbreviation 'cdc' in the older nomenclature (see Murray and Hunt, 1993). The primary CDK involved in the cell cycle control of fission yeast is encoded by the *cdc2*⁺ gene. In plants, nearly 50 homologues of the yeast *cdc2* gene have been cloned from several species, and comparison of the proteins encoded by their cDNAs has permitted a classification into five classes and a revised nomenclature (see Joubes *et al.*, 2000). Members of the CDK-a class share the PSTAIRE motif found in the cyclin-binding domain of yeast Cdc2, and most are able to rescue the temperature-sensitive *cdc2* mutant of fission yeast (for reference, 'CDK-a' is the nomenclature used to indicate plant CDKs of class A, whereas 'CDKA' indicates the protein product and *CdkA* the equivalent gene). CDK-b is a plant-specific group distinguished by the PPTALRE or PPTTLRE amino acid signature in their cyclin-binding domain, and is divided into the subgroups CDK-b1 and CDK-b2, according to which of these two possible amino acid motifs they possess. These CDKs are incapable of complementing the yeast *cdc2* mutation and display cell cycle phase specific expression, which is not observed for any CDKs in other organisms. At present, CDK-c, CDK-d and CDK-e contain fewer members. Members of the CDK-c class are marked by the PITAIRE motif and their function is poorly documented. CDK-d is represented by two plant sequences which share homology with kinases involved in activating phosphorylation of other CDKs (CDK activating kinases; CAK) and in activation of RNA polymerase II. CDK-e contains a single plant sequence with a SPTAIRE signature.

8.2.2.2 Cyclins

The first cyclin was originally discovered in extracts from fertilised sea urchin eggs, as a periodic protein that was synthesised throughout the cell cycle but degraded during mitosis (Evans *et al.*, 1983). Further examination suggested that cyclins are present in all eukaryotes. Furthermore, upon heterodimerisation with a CDK, an active serine/threonine protein kinase is constituted, consisting of the CDK as the catalytic subunit and the cyclin as the activating subunit. In plants, a diverse range of cyclins has been cloned and can be classified into nine groups based on sequence analysis: A1, A2, A3, B1, B2, D1, D2, D3 and D4 (Renaudin *et al.*, 1998). By analogy to other systems, it is hypothesised that specific cyclin/CDK combinations might confer substrate specificity or timing of activity. Subsequent interaction with other proteins, such as inhibitors, and post-translational modifications offer further mechanisms to control the activity of these kinase complexes. Some of the proteins involved in these additional regulatory mechanisms have been identified in plants.

8.2.2.3 Molecular control of cdk activity in the cell cycle

Several experiments have now demonstrated the role of specific CDKs in the progression through the cell cycle in plants, a role which is common to other organisms. Constitutive expression of a dominant negative mutation of *Arath*;CDKA;1 (*CDC2aAt* in the former nomenclature), resulted in a decrease of cell number in otherwise normal-sized plants. Treatment of cell suspension cultures with specific inhibitors of CDKA;1, such as oloumoucine and roscovitine, results in cell cycle arrest both at the G2/M and G1/S transition (Glab *et al.*, 1994; Planchais *et al.*, 1997). Constitutive expression of a dominant negative mutation of *Arath*;CDKB;1 (*CDC2bAt*), results in an increase of 4C cells in calli and cotyledons and a decrease of activity associated with this kinase (Mironov *et al.*, 1999). These data indicate that CDKB;1 activity is limiting for the duration of G2 or for the G2/M transition.

Mironov *et al.* (1999) have shown that the activity of CDK-a type complexes is highest during S-phase, G2 and M-phase and declines during G1. The activity associated with CDK-b-type complexes peaks during M-phase. However, our knowledge of the different kinase activities is still far from complete, since most assays use histone H1 as a substrate and factors with high affinity for CDKs such as p13^{suc1} and p9^{cks1Hs} for isolation of the complexes (for a discussion of these proteins see below in this section). However, it appears that not all CDK activity may be dedicated to the complexes that bind to these affinity matrices, and that histone H1 is probably not the first choice substrate for all of these complexes.

Regulation of expression levels of cyclins and CDK provides a coarse control on the kinase activity of CDKs involved in the cell cycle. During the cell cycle of cultured cells, cyclin A1 (*CycA1*) and A2 (*CycA2*) genes were expressed from S- to M-phase and *CycA3* genes were induced at the G1/S transition and decreased

prior to mitosis (Chaubet-Gigot, 2000). The strongest *CycB1* expression was associated with the G2/M transition. Ito *et al.* (1998) have shown that a nine base pair element in the promotor of this gene was responsible for this M-phase specific expression. Expression of *CycD*-type cyclins was induced in G1 and remains more or less constant in synchronised *Arabidopsis* cell suspensions during further progression of the cell cycle (Fuerst *et al.*, 1996). However, two D-cyclins from tobacco displayed an increase of their respective mRNAs during mitosis in a tobacco cell suspension, while a third did not (Sorrell *et al.*, 1999).

In plant organs, the different A- and B-types cyclins display cell cycle-dependent expression. Superimposed on this, three members of the cyclin B group of *A. thaliana* show tissue-specific expression patterns (Day and Reddy, 1998). In shoot tips of *Antirrhinum*, *CycD1* expression was present in the different tissues of the shoot, but mRNA levels were higher in meristems and vascular procambial tissues (Gaudin *et al.*, 2000). Furthermore, in this species, different *CycD3* genes were expressed in a tissue-dependent manner rather than in a cell-phase-dependent fashion. For example, in vegetative shoot apices, *CycD3a* is expressed in the peripheral regions of the meristem, whereas *CycD3b* is expressed in all proliferating tissue (Doonan, 1998; Gaudin *et al.*, 2000). *CycD4* expression was associated with specific phases of lateral root initiation, ovule development and embryo formation (De Veylder *et al.*, 1999).

In *A. thaliana* and in other plants studied, *Cdka;1* (*cdc2a*) expression was detected not only in dividing cells but also in cells with a 'high competence to divide' (Martinez *et al.*, 1992; Hemerly *et al.*, 1993). This term was used to describe cells that have the ability to resume division when an appropriate signal is received. Since this applies to many cell types in plants, CDK-a expression is rather widespread. It might be concluded that CDKA is not a limiting factor for division. In support of this, plants overexpressing the normal CDKA protein were not observed to have a specific phenotype, although a dominant negative form of the same protein inhibited cell division (see first paragraph of this section; Hemerly *et al.*, 1995). In contrast to the rather widespread expression of *Cdkas*, *Arath;Cdkb;1* (*cdc2bAt*) transcripts were distributed in a cell-cycle-dependent manner, which was reflected by a patchy distribution pattern when detected by *in situ* hybridisation experiments (Segers *et al.*, 1996).

In addition to cyclin binding, another level of regulation of the activity of cyclin-dependent kinases is imposed by phosphorylation. The stabilising and activating phosphorylation of the so-called T-loop is mediated by CDK activating kinases (CAKs) in most eukaryotes (Nigg, 1996). In plants, genes encoding this function have recently been cloned in *A. thaliana* and rice (Umeda *et al.*, 1998; Yamaguchi *et al.*, 1998). The kinase activity of these CAKs is positively regulated by cyclin H. Cyclin H seems to bind specifically to CAK and not to any other cyclin-dependent kinase (Yamaguchi *et al.*, 2000).

Full CDK activation requires removal of a phosphate group in order to allow binding of ATP. Inhibitory phosphorylation at the tyr-15/thr-14 residue is carried

out by the inhibitory kinase, Wee1, for which a maize homologue has been isolated (Sun *et al.*, 1999). Removal of this phosphate group and activation of the CDK requires homologues of the yeast phosphatase, Cdc25, for which no plant homologue has yet been found, although three genes exist in humans.

Association with another class of factor, CKS proteins, might offer a supplementary control of cdk activity. These proteins are found in all eukaryotes, including yeasts (p13^{suc1}) and humans (p9^{cks1}). In *Arabidopsis*, two CKS proteins have been isolated (De Veylder *et al.*, 1997; Stals *et al.*, 2000) that have high affinity towards most known CDKs, but their full function is still unresolved. They could act as assembly or docking factors, or perhaps mediate binding to positive or negative regulators, or be involved in protein degradation.

A family of CDK inhibitor proteins, the so-called CKIs, also mediates the activity of cyclin/CDK complexes. In *A. thaliana*, several genes encoding for these inhibitors can be identified in the genome, and the inhibitory activity of the proteins they encode has been demonstrated on some cyclin/CDK complexes (Stals *et al.*, 2000). So far, analyses of three different CKI encoding genes have been reported: *Cki1At* (previously known as *Ick1*; Wang *et al.*, 1998), *Cki2At* (previously *Ick2*; Lui *et al.*, 2000) and *Cki3At* (Stals *et al.*, 2000). Strikingly, these proteins display a sequence similarity of only 23 amino acids with the cyclin-dependent inhibitors of the Cip/KIP family in animals. Despite this low similarity, their inhibitory activity is conserved as has been shown both *in vivo* (CKI1At) and *in vitro* (CKI1At, CKI2At) (Wang *et al.*, 1998, 2000). Interestingly, CKI1At was shown to interact both with CDKA and CYCD3 via different regions (Wang *et al.*, 1998).

Degradation mediated by controlled proteolysis allows the cell to definitively remove regulatory proteins, such as cyclins, CKIs and structural proteins that form part of the cell cycle machinery. Some proteins are degraded via the ubiquitin-dependent pathway. Upon tagging of the protein with ubiquitin, they become a substrate for the 26S proteasome (Plesse *et al.*, 1998). Proteins that are putative targets for degradation via this pathway share a common structural motif in their N-terminal domain, the so-called D-Box. The protein levels of the mitotic A- and B-types cyclins (and thus their associated CDK activity) that carry this specific motif, appear to be under the control of ubiquitin-mediated proteolysis (Genschik *et al.*, 1998). Other cyclins, such as D-type cyclins, carry the PEST sequence. This sequence accounts for a rapid turnover of proteins in organisms such as yeast and mammals, but whether the same mechanism operates in plants is still unclear.

It is apparent that the fluctuation in the levels of specific proteins can differ from the expression patterns of the corresponding genes. Immunolocalisation experiments of cyclins and CDKs have provided a means to estimate the relative amount and intracellular location of each protein during the cell cycle. However, this approach does not provide information on the activity of the complexes formed.

In *Arabidopsis* cells at interphase, CDKA;1 was detected both in the nucleus and the cytoplasm. During late G2 and M-phase, CDKA;1 co-localised with the preprophaseband, and during prophase and metaphase CDKA;1 became associated with the mitotic spindle, and remained co-localised with the microtubule array during anaphase and telophase (Stals *et al.*, 1997).

In roots of *Zea mays*, the cyclin, ZmCYCB1;2, was detected only in dividing cells (i.e. actually in the process of division), whereas the presence of ZmCYCB1;1, ZmCYCA1;1 and ZmCYCB2;1 was related to tissues which contain dividing cells. ZmCYCB2;1 levels rose upon initiation of cell division and accumulated in the nucleus, indicating a possible role in the G0/G1 transition. This was followed by cytoplasmic accumulation of ZmCYCA1;1, coinciding with a reorganisation of the cortical microtubules (Mews *et al.*, 2000). Prior to mitosis, ZmCYCB1;2 and ZmCYCA1;2 are cytoplasmic, whereas ZmCYCB1;1 and ZmCYCB2;1 are nuclear. At the G2/M transition, ZmCYCB1;2 co-localises with the preprophaseband and with the nuclear envelope. ZmCYCA1;2 and the CDKA are also detected in the preprophaseband, and ZmCYCA1;2 is detectable on all microtubular structures during the cell cycle. ZmCYCB2;1 binds to the mitotic spindle and subsequently to the phragmoplast (Mews *et al.*, 1997).

8.2.2.4 D-type cyclins integrate extracellular signals and trigger the G1/S transition

The characterisation of D-type cyclins (Soni *et al.*, 1995) and elements of the 'retinoblastoma' pathway in plants has provided insights into how cell proliferation is triggered in the G1 phase. As discussed in section 8.2.2.2, D-type cyclins can be classified into four groups, D1, D2, D3 and D4. These proteins consist of about 300–350 amino acids, and are defined by the presence of an N-terminal motif consisting of the amino acids LxCxE (where x is any amino acid), which constitutes an interaction motif for the retinoblastoma protein (see below). This hallmark discriminates them from the other cyclins. Furthermore, all cyclins, including the D-type cyclins, contain a homologous region comprising about 100 amino acids that is involved in binding the CDK partner. Although this so-called 'cyclin box' has relatively low homology with the other plant cyclins, nine residues are conserved among them, and these include the five residues that are essential for catalytic activity. As stated above, almost all D-type cyclins also contain predicted PEST sequences, suggesting that these sequences may affect turnover of these proteins and consequently their effect on the cell cycle.

In synchronised cell suspension cultures of *A. thaliana* that are re-entering the cell cycle, the rise in *CycD3* expression has been correlated with the onset of S-phase, whereas *CycD2* expression is already detected in early G1 (Fuerst *et al.*, 1996; Riou-Khamlichi *et al.*, 2000). Both *CycD2* and *CycD3* mRNA levels responded positively to relatively low levels of glucose and sucrose. It was

demonstrated that this induction is not an indirect consequence of progression through the G1 phase of the cell cycle, but a more direct response to these sugars. Although these data were initially determined in suspension cultured cells, they were later confirmed in seedlings cultivated in liquid medium, in which it was shown that *CycD2* and *CycD3* expression is correlated with the availability of sucrose in the medium. In addition, *CycD3* expression is stimulated by cytokinins, but this induction also requires the presence of sugars. Of the different cytokinins tested (benzylaminopurine, kinetin, zeatin and isopentenyladenine), zeatin and benzylaminopurine were the most efficient at stimulating expression of *CycD3*. Overexpression of *CycD3* also conferred cytokinin-independent growth to calli (Murray *et al.*, 1998; Riou-Khamlichi *et al.*, 1999). The upstream events that link sugars and cytokinin levels to the expression of *CycD2* and *CycD3* are still not known. Nevertheless, it is now clear that the induction does not require additional protein synthesis and that a phosphorylation pathway is involved (Riou-Khamlichi *et al.*, 2000). These observations indicate that plant D-cyclins, by analogy with their animal counterparts, respond to extracellular growth factors. Interestingly, *CycD3* may lie at the intersection of different hormones affecting cell division, since brassinolide also induces *CycD3* expression and can replace cytokinins in culture media (Hu *et al.*, 2000).

The presence of the retinoblastoma-binding motif in D-type cyclins has led to the idea that retinoblastoma homologues are also present in plants and could be the target of D-type cyclins (Soni *et al.*, 1995). This hypothesis was further supported by the discovery that a geminivirus protein contains LxCxE, a retinoblastoma-binding motif (Xie *et al.*, 1995).

In animals, the retinoblastoma (RB) proteins and their relatives are the products of tumour suppressor genes, which act to prevent premature entry into S-phase by sequestering a variety of cellular proteins. As such, they could act as the molecular switch between cell proliferation and differentiation. The RB proteins are also targeted by oncogenic DNA viruses, which require host DNA synthesis for their own replication. RB proteins bind homologues of the yeast MSI protein, which is proposed to be involved in chromosome organisation and the regulation of the Ras-cAMP pathway that controls cellular growth. They also interact with D-type cyclins, and bind transcription factors, such as E2Fs, which activate S-phase genes. The binding of RB to E2F results in the recruitment of the RB protein to promoters containing E2F-binding sites. The binding of the protein then attracts histone deacetylase activity to these promoters, resulting in chromatin reorganisation, and so making the DNA less accessible to the transcriptional machinery. Upon the interaction of the CyclinD/CDK complexes with the RB protein and subsequent phosphorylation of RB by these active kinases, the protein loses its association with E2F, leading to activation of E2F-regulated genes and entry into S-phase (Bartek *et al.*, 1997; Lavia and Jansen-Dürr, 1999).

With the isolation of RB homologues in different plant species, it became possible to determine whether a similar mechanism for control of the G1/S transition exists in plants. Comparison of the amino acid sequences of RB-related proteins revealed that the 'pocket' domain, necessary for binding of several cellular proteins including E2F and D-type cyclins, is conserved among animals and plants. Retinoblastoma proteins from maize have been shown to interact with plant CYCD3 type cyclins but not with a mitotic cyclin (Graf *et al.*, 1996). Both *Arabidopsis* CYCD2 and CYCD3 bind to the maize retinoblastoma protein, and this binding is dependent on the presence of the cyclin LxCxE binding motif. Furthermore, mammalian cyclin/CDK complexes phosphorylate the RB protein of maize (ZmRB). The ZmRB protein was also found to be capable of repressing E2F-dependent transcription (Huntley *et al.*, 1998). In addition, a complex consisting of tobacco cyclin D3 and p34CDKA was capable of phosphorylating tobacco retinoblastoma protein *in vitro* (Nakagami *et al.*, 1999). It would be interesting to determine whether all these cyclin/CDK complexes display the same affinity for the retinoblastoma proteins.

Genes encoding plant homologues of E2Fs have recently been cloned in several species (Ramirez-Parra *et al.*, 1999), and in *Arabidopsis*, three homologues have been identified (de Jager, Menges, Bauer and Murray, 2001). Moreover, putative E2F-binding sites were identified in genes involved in DNA replication and the G1/S transition, such as the ribonucleotide reductase and cyclin D3 promoters.

In summary, these observations suggest that in plants, D-type cyclins integrate signals, such as nutrient status and phytohormones, during the G1 phase and transduce these signals via the retinoblastoma pathway. Cyclin-dependent phosphorylation of RB proteins then results in the release of factors that promote entry into S-phase.

8.2.2.5 Plant hormones and the cell cycle machinery

Plant hormones influence cell proliferation but our knowledge of their mode of action in this process is still fragmentary. As an additional difficulty in the research on their mode of action regarding the cell cycle, plant hormones have been shown to affect a wide variety of processes (Davies, 1995). Therefore, the observations made *in planta* might be secondary rather than direct effects on the cell cycle. Moreover, a treatment that induces cell division will, by definition, result in activation of cell division related genes, even if these are not directly induced by the hormones. Furthermore, treatment of plant cell cultures with specific concentrations of these plant growth regulators might elicit responses as a consequence of other signal transduction pathways not related to the cell cycle. Experiments indicating cell cycle effects of hormones must therefore be rigorously examined if the effects are to be considered direct.

In deepwater rice, gibberellin (GA) treatment results in accumulation of mRNAs of a gene with 55% sequence identity to a CDK-activating kinase.

a *CdkA* gene (*cdc2OS-2*) and two mitotic cyclin genes (Sauter, 1997). These results indicate that GA can induce cell division in this system, but not necessarily that these genes are under direct GA control.

Cytokinins and auxins are indispensable to maintain undifferentiated cells in proliferation during culture *in vitro*. Although their mode of action in respect to the cell cycle machinery is far from being understood, some data have emerged. In roots, treatment with cytokinins or auxins increases the expression of *CdkA* (*cdc2At*) in a tissue-dependent manner, whereas they act synergistically in protoplasts. Cytokinins promoted expression in the pericycle and parenchyma cells of the vascular strand in the upper part of the main root. Auxin treatment transiently induced expression in a tripartite pattern. After two days of treatment, root tips showed strong expression in two zones that were separated by a zone of weaker expression. This characteristic pattern disappeared after four days in the presence of auxin (Hemerly *et al.*, 1993). These effects are relatively long-term changes observed after treatment, and are therefore likely to be caused as indirect consequences of changes induced by hormone treatment.

The genes involved in auxin responses, such as *Tir1* and the *Axr*, seem to be linked to ubiquitin-mediated processes. *Axr1* encodes for a protein related to the ubiquitin-activating enzyme (Leyser *et al.*, 1993), and TIR1 shares characteristics of proteins being part of the ubiquitin-protein ligase complex (Ruegger *et al.*, 1998). It is an attractive idea that auxin would influence cell proliferation by affecting protein turnover.

Cytokinins have been reported to influence different phases of the cell cycle in a variety of models. A detailed review by Jacquemard *et al.* (1994) suggests that effects have been observed on the G1/S and G2/M transitions, as well as progression through S-phase.

Most of these studies have been conducted by supply or removal of cytokinin from the growth medium of cell cultures. In an attempt to understand the role of cytokinins in cell cycle progression, the endogenous levels of cytokinins have been determined in a synchronised cytokinin-autotrophic tobacco cell suspension line (BY-2). The only fluctuating cytokinins were cytokinins of the zeatin type, which showed a transient accumulation at the end of S-phase and early M-phase (Redig *et al.*, 1996). The effect of depleting endogenous cytokinin levels on cell cycle progression in this model was studied by treatment with lovastatin (Laureys *et al.*, 1998, 1999). Lovastatin, a competitive inhibitor of 3-hydroxy-3 methylglutaryl (HMG)-CoA reductase, reduces the formation of mevalonic acid, itself a precursor for the biosynthesis of a precursor of isoprenoids. Lovastatin treatment resulted in a decrease of endogenous isoprenoid-derived cytokinins, and blocked the cells at the G2/M transition. Of the different isoprenoid and aromatic cytokinins tested, only zeatin could restore this arrest. This observation on the specific role of zeatin in the cell cycle may be linked with results obtained by immunolocalisation in tobacco apices, where zeatin was the only cytokinin base out of three isoprenoid cytokinin bases that accumulated

in the nucleus (Dewitte *et al.*, 1999). Furthermore, this lovastatin-mediated cytokinin starvation did not prove restrictive for the G1/S transition (Laureys *et al.*, 1999). Similarly, in a cytokinin dependent *Nicotiana plumbaginifolia* cell suspension culture, auxin treatment of cells (after auxin and cytokinin depletion for several days) resulted in a cell population with a DNA content higher than observed in G1 cells. This was interpreted as an arrest in G2 due to the lack of cytokinins. Nevertheless, benzylaminopurine, an aromatic cytokinin, induced dephosphorylation (and thus activation) of a CDKA-like protein in *N. plumbaginifolia* cells in G2 and in tobacco pith cells grown in the presence of auxin (Zhang *et al.*, 1996).

CDK complexes are inactivated by phosphorylation of Thr-14 and Tyr-15 residues, and progression into mitosis depends on removal of these inhibitory phosphate groups by Cdc25-like phosphatases. It is an appealing hypothesis, therefore, that cytokinin mediated-dephosphorylation triggers G2/M transition. From the data discussed above, zeatin would be the first candidate for an endogenous cytokinin capable of fulfilling this role. However, it still remains to be determined whether endogenous cytokinins, such as zeatin, indeed promote dephosphorylation of CDKs, and whether this specific phosphorylation is limiting for the G2/M transition. In addition, it will be interesting to determine whether or not the promotion of phosphorylation is the result of the stimulation of the activity of endogenous phosphatases. Alternatively, cytokinins could downregulate the activity of the WEE1 kinases or promote CDK phosphorylation through a more indirect route. Furthermore, it will be interesting to determine whether transient cytokinin signals integrate extracellular signals, or whether they link intracellular processes.

The unique role for cytokinins in the G2/M transition in cell suspension cultures appears to contradict the previously discussed stimulatory effect of cytokinins on *CycD3* expression. However, it is not clear whether the kinase activity declined to a critical level needed for G1/S arrest in these cytokinin starvation experiments in cell suspension cultures. This may be particularly relevant, since these cell cultures are selected for a high proliferation capacity and the experiments are performed in the presence of optimal concentrations of nutrients and auxin, factors to which *CycD3* is also responsive. Moreover, the selection process involved in generating these continuously proliferating cell cultures may have resulted in a partial or complete loss of G1 controls, which would normally regulate the re-entry of quiescent cells into the cell cycle. It is possible that cytokinin action during the G1/S transition might also be coupled to the activation of replicon origins. In *Sinapis*, a treatment with benzyladenine, an aromatic cytokinin, resulted in an increase in the number of replicon origins (Houssa *et al.*, 1990). The role of CYCD3 in this process is still speculative but worth investigating.

The study of mutants that link sensitivity towards cytokinins with cell division is expected to be particularly useful in revealing the signal transduction pathway

which connects cytokinin sensing to the cell cycle machinery. Recent studies have implicated two-component histidine kinases in cytokinin signalling and detection (Kakimoto, 1996; Inoue *et al.*, 2001). Several cytokinin hypersensitive (Kubo and Kakimoto, 2000) and resistant mutants (Jullien *et al.*, 1992; Faure *et al.*, 1994) have been identified. Furthermore, dwarfed mutants have been screened for their sensitivity towards various hormones. In this way, it was established that the *Stunted plant 1* gene mediates a cytokinin effect on cell division in roots (Beemster and Baskin, 2000).

Brassinosteroids, a class of steroid hormones present in plants, were shown to be limiting for cell elongation, male fertility, senescence and xylogenesis. Their role in the cell cycle is still contradictory (Clouse and Sasse, 1998). Recently, by screening a cDNA array it was found that brassinosteroids upregulate *CycD3*. Moreover, they could substitute for cytokinins in callus and suspension cultures of *Arabidopsis*. This induction of *CycD3* expression requires *de novo* protein synthesis but is not phosphorylation dependent (Hu *et al.*, 2000). This finding reinforces the argument that *CycD3* expression can respond to a variety of extracellular conditions.

Absciscic acid has been described as a repressor of cell division. This plant hormone has been shown to induce the expression of a gene encoding for the cyclin-dependent kinase inhibitor, *Cki1At*, leading to a decrease in the kinase activity associated with CDKA (Wang *et al.*, 1998).

8.2.3 *The symphony: cell cycle progression via regulation of the activity of cyclin-dependent kinase complexes*

An attempt to integrate data from a variety of sources is presented in figure 8.1. The commitment to cell division may be initiated when expression of D-type cyclins is increased by extracellular signals, such as hormones and sugars. The D-cyclins bind to the CDKAs and this activated complex phosphorylates the RB proteins. This results in the liberation of E2F and thereby triggers entry into S-phase and promotes the expression of genes involved in DNA replication. D-type cyclins are degraded by proteolysis or out-competed by A-type cyclins, which form an active complex with the CDKA during S-phase until the G2/M transition (Roudier *et al.*, 2000). The targets of this complex are still unknown. In *Arabidopsis*, downregulation of CDKB lengthens the G2 phase (Mironov *et al.*, 1999), indicating a role for CDKB in progression through G2 phase or entry into M-phase. A checkpoint for the completion of DNA replication is likely to occur during G2 before the cells enter the M-phase. The concentration of zeatin-type cytokinins is limiting for this transition. CDKs are dephosphorylated at their catalytic cleft in late G2, and a putative role for zeatin-like cytokinins in this process is postulated. During entry into mitosis, it seems that the serine/threonine specific phosphatases control the coordination of chromosomal and microtubular organisation. In addition, the phosphatases

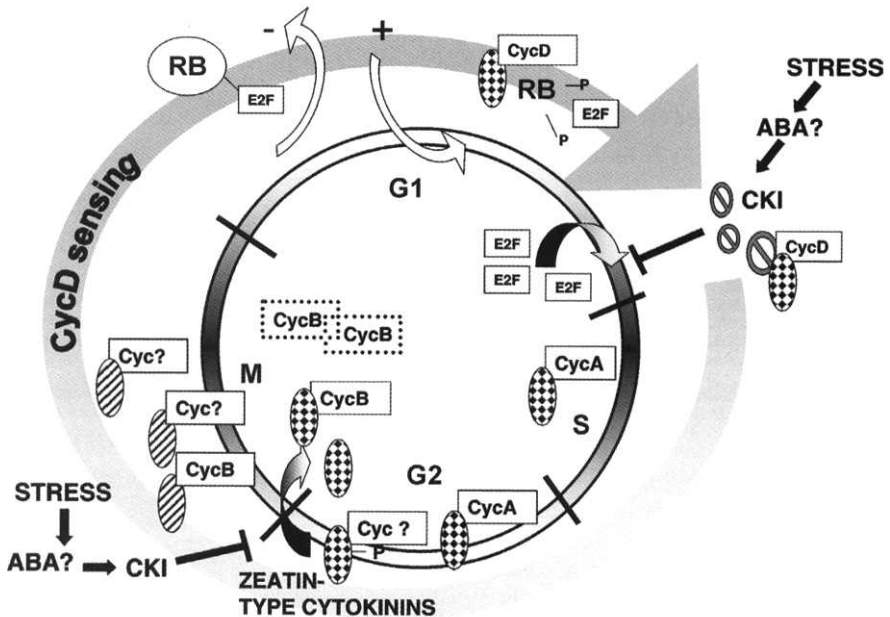


Figure 8.1 Overview of cell cycle progression in higher plants, driven by active cyclin-dependent kinase (CDK) complexes. Favourable extracellular conditions, such as sufficient levels of hormones and carbon source, stimulate expression of genes coding for D-type cyclins. D-type cyclins bind to CDKAs (chequerboard-filled ellipses) and this active complex phosphorylates retinoblastoma (RB)-related proteins, resulting in the release of transcription factors (E2F). The E2Fs trigger expression of genes involved in DNA replication and entry into S-phase occurs. During S-phase and G2, CycA/CDKA complexes are formed. Entry into M-phase is triggered by the activating dephosphorylation of CDKA, and it is postulated that cytokinins play a role in this process. M-phase progression is associated with active CDKA and CDKB (striped ellipses) complexes, and it has been proposed that these complexes play a role in reorganisation of microtubular structures and phosphorylation of nuclear proteins. Exit from mitosis requires degradation of B-type cyclins. If levels of CYCD-type cyclins fall below threshold, exit from the cell cycle will occur. Stress conditions, which may threaten the ability of the cell to complete cell division, can cause an arrest in G1 or G2, possibly via the action of cyclin-dependent kinase inhibitors, whose expression can be triggered by abscisic acid (ABA).

controlling the dephosphorylation of the cyclin-dependent kinases (Cdc25-like) might be regulated by these phosphatases (Ayaydin *et al.*, 2000). It is proposed that CDKAs, in complex with B-type cyclins, phosphorylate nuclear proteins to trigger the entry into mitosis (Mironov *et al.*, 1999).

In alfalfa, kinase activity associated with CDKA declines during the M-phase after a transient peak during G2. In the same species, the activity linked with one type of CDKB (CDC2MsD) remains constant during most of the M-phase, whereas another CDKB (CDC2MsF) is clearly specifically activated during M-phase. Similar observations have been made in *Arabidopsis*. The activity of a

CDKB in *Arabidopsis* peaked early in M-phase, whereas gene expression was predominantly associated with G2 (Mironov *et al.*, 1999). Recently, a second CDKB similar to CDC2M_sF has been identified that shows M-phase specific expression (Murray, unpublished data).

The composition of all the CDKA and B-complexes is not known, nor are their substrates. Data obtained by immunolocalisation indicate that CDKA in *Arabidopsis* accumulates on the preprophase band, on the mitotic spindle, phragmoplast and chromosomes (Stals *et al.*, 1997; Mészáros *et al.*, 2000). In alfalfa, CDKB was detected on the same cytoskeletal structures, but not on the chromosomes (Mészáros *et al.*, 2000). In view of the results obtained by immunolocalisation of cyclins in *Zea mays* (Mews *et al.*, 2000), it is likely that the different complexes of CYCAs/CDKA,B and CYCBs/CDKA,B fuel processes such as pre-prophaseband assembly, nuclear envelope degradation, degradation of the pre-prophaseband and dynamics of the mitotic spindle by phosphorylation of nuclear proteins and proteins associated with the microtubuli. The activities of both CDKAs and CDKBs decrease at the end of M-phase (Mészáros *et al.*, 2000). Furthermore, it is essential for a proper progression through the last phases of mitosis that cyclins, such as cyclin B, are degraded by proteolysis (Genschik *et al.*, 1998).

8.3 The complete oeuvre: cell division, plant growth and development

Overexpression of cell cycle genes can result in an overall shortening of the cell cycle or a reduction in length of a specific cell cycle phase. The latter may be compensated by an increase in the duration of other phases. Furthermore, if the balance between the growth cycle and cell division is disturbed in favour of cell division, one might expect smaller cells, unless cell growth is stimulated equivalently. In contrast, downregulation of cell cycle genes could induce opposite effects. These may include longer cell cycles and increases in length of one specific phase, possibly counteracted by a shortening of the other cell phases. Also, bigger cells may develop. Since the development of an organism requires strict control of cell division, there may also be effects on the morphology, such as ectopic cell divisions leading to changes in differentiation patterns.

The constitutive overexpression of *CdkA* (formerly *cdc2a*) in *Arabidopsis* had no apparent phenotype, although CDK activity was increased. In contrast, titrating out the active CDKA by constitutive overexpression of an inactive (and thus, dominant negative) CDKA was lethal in *Arabidopsis* and resulted in a decreased number of larger cells in tobacco. In this heterologous tobacco system, plants overexpressing the mutant CDKA protein were slightly smaller than wild-type. Interestingly, cells of all tissues, except cells in the flowers and mature embryos, were larger in the mutant when compared to wild-type. The

anatomical structure of the plants was not significantly altered, and nor was the rate of development modified by overexpression of the mutant *CdkA* (Hemerly *et al.*, 1995). These results might imply that cell growth is not only linked to the cell cycle but also controlled by other developmental mechanisms that can compensate for a reduced rate of cell division. Alternatively, the larger cells could result from an uncoupling of cell growth and cell division; that is, since the latter was inhibited, cell growth continued to result in larger cells.

The constitutive overexpression of an *Arabidopsis* cyclin-dependent kinase inhibitor, *Cki1*, produced similar effects. Growth was severely inhibited and most organs of the transgenic *Arabidopsis* plants were smaller. Moreover, the overall phenotype of the plant was profoundly altered by the overexpression of the inhibitor. In common with the results obtained with the dominant negative CDKA mutation, cell size was increased in the transgenic. Most of the effects could be linked to a reduction in cell division but some others, such as precocious flowering and a reduction in apical dominance, are more difficult to explain (Wang *et al.*, 2000).

Constitutive overexpression of yeast phosphatase, *cdc25*, in tobacco was reported to inflict precocious flowering, twisted and wrinkled vegetative leaves with an extended lamina and smaller cells in lateral roots (Bell *et al.*, 1993). Inducible overexpression resulted in an increase of lateral root initiation and a decrease of cell size in these root primordia. It was suggested that overexpression of yeast *cdc25* resulted in an excessive dephosphorylation, and thereby activation, of CDKA. However it is also possible that yeast Cdc25 might affect more than one target in plant cells (McKibbin *et al.*, 1998).

Inducible downregulation of the *Arabidopsis Cdkb1;1* gene by expression of an antisense mRNA resulted in a decrease of cell size in epidermal hypocotyl cells in seven-day-old etiolated seedlings. Furthermore, in dark-grown antisense *Cdkb;1* seedlings, the differentiation of the tissues of the hypocotyl was inhibited. However, the cell number and size of the shoot apical meristem proved to be unaffected by the induced antisense construct. Remarkably, cells in the meristems of antisense transgenic seedlings proved to be more highly vacuolated, which might indicate a mild positive effect on the differentiation of these cells (Yoshizumi *et al.*, 1999). These results indicate that CDKB1:1 action during this phase of development is most critical for the regulation of hypocotyl cell elongation in etiolated seedlings (Yoshizumi *et al.*, 1999). The lack of cell cycle activity in the shoot apical meristem during the first seven days in dark-grown seedlings (Hemerly *et al.*, 1993) might explain the lack of effect in antisense plants. However, the absence of an obvious effect on the cells of the shoot meristem under light conditions is certainly intriguing, especially since these antisense plants had smaller cotyledons and leaves when compared with control plants of the same age. A more detailed cell cycle analysis of these plants might, therefore, be revealing, together with confirmation that CDKB was indeed downregulated in meristem cells. If the protein is downregulated,

these results would suggest that CDKB action during this phase of development is critical for the regulation of hypocotyl cell elongation in etiolated seedlings.

The overexpression of a mitotic cyclin, *CycB1*, throughout the cell cycle increased the growth rate of roots in *Arabidopsis*. This promotive effect was explained by an increase in cell production rather than by an increase in cell growth (Doerner *et al.*, 1996).

Expression of the *Arabidopsis* *CycD2* gene in tobacco promoted the growth rate, as characterised by a higher rate of leaf initiation, an increased aerial biomass accumulation and a more rapid attainment of flowering size. No size difference in mature cells were observed between transgenics and wild-type. Therefore, faster growth could only be the consequence of enhanced cell division, and this was attributed to a shortening of the G1 phase and an increase of the proportion of cells in division through the overexpression of the *CycD2;At*. From these results, it was concluded that the growth rate can be regulated by shortening the G1 phase, and that this process can be mediated by control of the expression of *CycD2* cyclins (Cockroft *et al.*, 2000).

Constitutive overexpression of *CycD3* in *Arabidopsis* resulted in wrinkled leaves and retarded growth. Flowering occurred with the same number of leaves when compared with the wild-type, but cells in shoot meristems were smaller and the meristems disorganised (Riou-Khamlichi, Dewitte, den Boer and Murray, unpublished data).

Parameters such as temperature and CO₂ concentration affect growth by influencing cell division. Optimal temperatures, as compared to hypo- or supra-optimal temperatures, result in shorter cell cycle times, and the largest effect is in G1 (Francis and Barlow, 1988). An increase in ambient CO₂ concentration induces a higher proportion of cells to cycle in the grass, *Dactylis glomerata* (Francis, 1998).

These data clearly demonstrate that various genes affect different aspects of the cell cycle in plants. To a certain extent, it also illustrates the flexibility of plant development towards cell numbers, indicating that positional information governing differentiation and organogenesis is not strictly dependent on cell numbers. This aspect of the relationship between cell division and positioning of differentiation was recently underlined by the expression of the dominant negative CDKA protein in *Arabidopsis* under the control of a late-heart-stage embryo specific promoter. During this phase of development, cell division was shown to be necessary in order to create the embryo structure and the apical-basal pattern. However, even in severe phenotypes, radial cell differentiation was little affected (Hemerly *et al.*, 2000).

Furthermore, the overall growth rate of the plant appears to be dependent on cell division, since inhibition of the cell cycle by downregulation of CDK activity results in smaller plants. The loss of cell numbers is only partially compensated by cell growth. Secondly, overexpression of D2-type cyclins results in a faster cell cycle, which is reflected by faster growth.

These data also indicate that cell cycle genes might also be involved in other processes, direct or indirect, such as cell expansion and apical dominance.

8.4 Contextualisation: reflections on cell cycle regulation in the context of plant development

In shoot meristems, cell divisions are not synchronised between neighbouring cells, and cells with an extremely slow cell cycle may be present in all regions of the meristem. However, in general, cell division activity is zoned in meristems. In shoot meristems, cell cycle times were found to be shorter in the peripheral zone compared to those in the central zone. The mean cell generation time is higher in the summit than in the flanks (Lyndon, 1998). Furthermore, planes of division seem to be coordinated, and the L1 and L2 layers that form the tunica are marked by a low frequency of periclinal divisions and a predominance of anticlinal divisions. Initiation of primordia is accompanied by a rise in periclinal divisions, with the initial periclinal division probably acting as a trigger for the formation of a new primordium.

8.4.1 What is the function of this distribution of cell division?

Firstly, the dome shape of root and shoot meristems might be a direct consequence of the presence of slower cycling cells in the central zone of these structures. Architecturally, a dome is a strong structure which can protect the underlying cells, and in the case of the root can resist pressure in order to allow penetration of the substrate. Secondly, clonal analysis of shoots in *Arabidopsis* revealed that all cells originate from one group of stem cells (Furner and Pumfrey, 1992; Irish and Sussex, 1992). These slow-growing cells in the central zone of the shoot tip can therefore act as a reserve of stem cells. In addition, these experiments revealed that there are no restrictions on cell fate within the different cell layers of the shoot meristem in seeds (Furner and Pumfrey, 1992).

8.4.2 What controls the cell cycle in the shoot apical meristem?

This has been a topic of research for half a century now and is still largely unresolved. If the control of cyclin D gene expression is examined, which is almost certainly an oversimplification, can we make some educated guesses? *CdkAs* are expressed throughout the meristem, and this has been interpreted as being associated with the tissue's competence to divide. *CdkBs*, however, are expressed in a patchy manner, consistent with their expression during G2/M transition. The *Arabidopsis CycD3;1* gene is expressed almost uniformly in all dividing tissues of the meristem and the vascular tissues. However, in *Antirrhinum* two structurally similar genes, *CycD3a* and *CycD3b*, display strikingly different

expression patterns (Gaudin *et al.*, 2000). Whereas the expression of *CycD3b* is present in all actively dividing cells, *CycD3a* is expressed in young primordia and vascular tissue. In particular, *CycD3a* expression is not observed in the main part of the meristem, which may indicate the existence of a gradient in CYCD3 proteins levels being generated by the activity of two promoters under different developmental controls. It is possible that *CycD3a* expression is downregulated by some factors generated by the central zone; the full activation of the *CycD3a* gene could only take place in cells further away from the central zone. As auxin was indicated as a determining factor of initiation and radial patterning of lateral organs (Reinhardt *et al.*, 2000; see also chapter 6 of this volume), it will be interesting to see whether or not the expression of these D-type cyclins is a target of auxin action. Expression of *CycD3b* alone might be sufficient to maintain a threshold in kinase activity, resulting in lower cell cycle activity. Furthermore, as the retinoblastoma protein is proposed to maintain a differentiated state in animals (Zhu and Skoultchi, 2001), this threshold kinase activity could be sufficient to prevent differentiation in the central zone.

In the context of cyclin D control of the cell cycle in the shoot meristem, another remarkable observation was made in the shoot apical meristem of *Antirrhinum* (Gaudin *et al.*, 2000). New floral primordia and sepals were observed as separated from the other parts of the meristem by a discontinuity in *CycD3b* expression. This localised decrease of *CycD3b* expression corresponds to approximately three cell layers, and may reflect a local cell cycle inhibition allowing the new buttress to grow out from the surface of the dome of the meristem. Besides this mechanical interpretation, the *CycD3b* expression pattern might also reflect a developmental boundary, associated with the separation of the specific gene products functional in the meristem and the primordium. In *Arabidopsis* seedlings, the cells at the boundary between the apical meristem and the cotyledons express the *CUC2* gene, which indicates that the *CUC2* gene has a role in the detachment of lateral organs (Aida *et al.*, 1999). Could *CUC2* be linked to the downregulation of cell cycle activity? Is loss of *CycD3b* expression indicative or causative in this effect? Such questions await further investigation.

Several homeotic genes that regulate the development and organisation of the meristem have been identified by mutagenesis (for recent reviews see Lenhard and Laux, 1999; Bowman and Eshed, 2000; see also chapter 2 of this volume). As cell division is a putative target for their action, it is examined further in this section, which looks at the relationship between the *SHOOT MERISTEMLESS* (*STM*) gene and the *CLAVATA* (*CLV*) genes in the *Arabidopsis* shoot meristem in more detail. The *STM* gene is expressed throughout the meristem but not in the primordia. Mutations in this gene block formation of the shoot meristem in seedlings, and result in partly fused cotyledons. Strong alleles occasionally develop a single leaf postembryonically and weaker alleles develop numerous leaves in between the two cotyledons, as well as rosettes of leaves

and inflorescence stalks. Such weak alleles characteristically develop fewer flowers, and the flowers that are produced have a reduced number of central organs.

The action of the *STM* gene is antagonised by the genes of the *CLAVATA* pathway, encoded by three genes *CLV1*, *CLV2* and *CLV3*. A mutation in any of these *CLAVATA* genes results in the accumulation of cells in the meristem. It has been proposed that *STM* function is required for appropriate high rates of cell division in vegetative and floral meristems (Meyerowitz, 1997). Alternatively, the function of *STM* has been interpreted as promoting or maintaining the undifferentiated state of cells in the central zone of the meristem (Clark *et al.*, 1996; Endrizzi *et al.*, 1996). From the observations that *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate the development of the shoot meristem, it was proposed that these genes play opposing roles in the regulation of cell division and cell differentiation (Clark *et al.*, 1996). Our understanding of the biochemical nature of differentiation is still vague, making it hard to evaluate the state of differentiation of a tissue or a cell in the absence of visible criteria. Alternatively, *STM* could be the clutch between the gearbox and the cell cycle engine, uncoupling participation in a morphogenetic programme from cell division or, as formulated by Lenhard and Laux (1999), *STM* prevents cells from participating in organ formation.

Since *CLAVATA* genes antagonise the action of *STM*, encode a ligand receptor pathway and are expressed in overlapping regions of the central zone, their action in this zone is expected. In addition, the *WUSCHEL* (*WUS*) gene is required for the establishment of the shoot apical meristem and to maintain the undifferentiated state of the stem cells (Laux *et al.*, 1996; Mayer *et al.*, 1998). It was therefore proposed that the population of stem cells in the central zone is regulated by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. In this system, *WUS* expression is sufficient to induce meristem cell identity, whereas the *CLAVATA* pathway stimulates organ formation. This is underlined by the striking similarities in the phenotype of the weaker *CLAVATA3* overexpressors and the *WUS* mutant (Laux *et al.*, 1996; Brand *et al.*, 2000). In both cases, numerous ectopic leaf meristems are initiated across the terminated apex, including the central zone, from which primordia initiation are excluded in the wild-type.

These data indicate that the link between cell division and *STM*, *CLV* and *WUS* action may be more indirect. It therefore seems that these homeotic genes influence the developmental context rather than exerting a direct action on cell division. However, other candidates for a more direct action on cell cycle are beginning to emerge. A set of homeobox genes of tobacco has been cloned, the expression pattern of which matched the cytohistological zonation of central zone and peripheral zone. However, their function is still unclear (Nishimura *et al.*, 1999).

8.5 Conclusion

Cell cycle entry and progression are mediated by the activity of cyclin-dependent kinases. Data are emerging concerning the mechanisms controlling their action in the cell cycle. The D-type cyclins respond to the nutrient status and phytohormones, and trigger the G1/S transition. Cell division may be a key factor in determining growth rate but development, on the other hand, has certain flexibility towards cell numbers. It is anticipated that the control of cell division in meristems includes a complex set of interactions between homeotic gene products, hormonal signalling and environmental sensing. These links are now emerging and are expected to be a major research topic in the future.

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9 The root meristem

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9.1 Introduction

Meristems are centres for the production of new cells and organs throughout the life of the plant. The meristem of the primary root of most angiosperms is laid down during embryogenesis, and continues to be active for variable times after germination and the emergence of the root from the seed coat. Post-germination development involves the maintenance of the patterned array of cells that is set up during embryogenesis. Cells in the pericycle of the primary root develop new root meristems, which develop into lateral roots that will go on to form the complex root system. This chapter describes progress towards elucidating the mechanisms that underpin pattern formation in the embryo, and the maintenance of pattern in the post-germination root meristem.

Arabidopsis has been used extensively as a model system for the study of the root meristem for a number of reasons. The root is small (*ca.* 80 μm in diameter) and has a simple cellular organization (Dolan *et al.*, 1993). The small size of the root makes it amenable to microsurgical manipulation using lasers. Cell-to-cell signalling can be studied, using lasers that destroy a target cell or group of cells, and the developmental consequences assessed together with their effect. Large numbers of mutations can be isolated by visual screening of 3–5 day old seedlings grown in petri dishes. These mutations can be mapped easily and the corresponding wild-type gene cloned and sequenced. The availability of the entire genome sequence for *Arabidopsis* has accelerated the speed at which such walks can be completed and provides useful tools with which to examine gene function during root development. This chapter provides a description of *Arabidopsis* root anatomy and its development in the embryo. It also describes different classes of mutants impaired in different aspects of meristem formation/function that provide insights into the mechanism of root development.

9.2 Embryonic ontogeny of the *Arabidopsis* root meristem

Embryogenesis in *Arabidopsis* leads to the formation of two populations of stem cells from which the body of the mature plant is derived, the shoot and the root meristems. These meristems are located at opposite ends of the embryo, with the root meristem forming at the basal pole of the apical-basal axis. The

Image Not Available

Figure 9.1 Embryonic origin of the *Arabidopsis* root meristem. The asymmetric division of the zygote yields a small apical cell and a larger basal cell. In the globular stage embryo, stereotypic divisions of the apical cell lead to the formation of protoderm, ground and vascular tissue. Transverse divisions of the basal cell yield the hypophysis and the suspensor. Transverse division of the hypophyseal cell leads to the formation of the quiescent centre and columella initial. In the early heart stage embryo, all initials of the different tissues are present surrounding the quiescent centre. The contribution of the different embryonic tiers and initials to the seedling root meristem can be followed by referring to the key. Abbreviations: lrc, lateral root cap; cort/endo, cortex/endodermis. (Reprinted with permission from B. Scheres, Utrecht University, The Netherlands.)

root meristem develops from derivatives of both the apical and basal cells of the two-celled embryo, which form as a result of an asymmetric cell division of the elongated zygote (figure 9.1). Transverse divisions in the basal cell gives rise to cells that develop into the extra-embryonic suspensor. The apical cell goes on to form the embryo proper. The root meristem forms from cells on either side of the suspensor/embryo proper boundary. The upper cell of the suspensor will go on to form the central cells of the quiescent centre and the columella (central part of the root cap). The remainder of the meristem is derived from the basal-most derivative of the apical cell (two-celled embryo). From the octant to heart stage, a regulated pattern of cell divisions leads to the formation of the root promeristem. By the late heart stage, all the initials of the different tissues of the root are present and surround the quiescent centre (figure 9.1).

9.3 Organization of cells in the primary root of *Arabidopsis*

The primary root of *Arabidopsis* can be divided into three organizational zones along its longitudinal axis. At the base of the root, the meristem comprises dividing cells and a template of initials which are ensheathed by the root cap. Above the meristem is a zone where the cells grow in the absence of cell division, termed the elongation zone. When cells have stopped elongating, they undergo the final stages of maturation in the differentiation zone (figure 9.2A).

Image Not Available

Figure 9.2 Organization of the *Arabidopsis* primary root. **A:** Scanning electron micrograph (SEM) of the root showing the different zones of the root. Abbreviations: M, meristematic zone; E, elongation zone; D, differentiation zone. **B:** SEM of the root at the beginning of the differentiation zone. The alternating files of trichoblasts (T) and atrichoblasts (AT) are highlighted with arrows. The tubular outgrowths from trichoblast cells are root hairs. **C:** Cross-section through the meristematic zone of a 3-day old *Arabidopsis* root showing the concentric rings of the different tissues. The section is stained with toluidine blue. Abbreviations: LRC, lateral root cap; EP, epidermis; C, cortex; EN, endodermis; V, vasculature; AT, atrichoblast; T, trichoblast. (The SEM images, A and B, were kindly provided by K. Findlay and P. Linstead, respectively. The cross-section, C, was provided by P. Linstead. Kim Findlay and Paul Linstead are affiliated to John Innes Centre, Norwich, UK.)

In the radial dimension, cell types are organized as concentric rings around the stele, where the vasculature (phloem and xylem) is located. The lateral root cap is the outermost layer. The epidermis is located inside the lateral root cap and outside the cortex, which in turn lies outside the eight cells of the endodermis. The next layer is the pericycle, from which lateral roots originate (figure 9.2C).

In the growing root, all cells are derived from initials that surround the four central cells of the quiescent centre (figure 9.3). Initials divide in a stereotypical pattern to give rise to the cells in each of the tissue layers. For example, lateral root cap and epidermis originate from a single protodermal initial that divides periclinally. The cortical initial divides first anticlinally to regenerate a lower

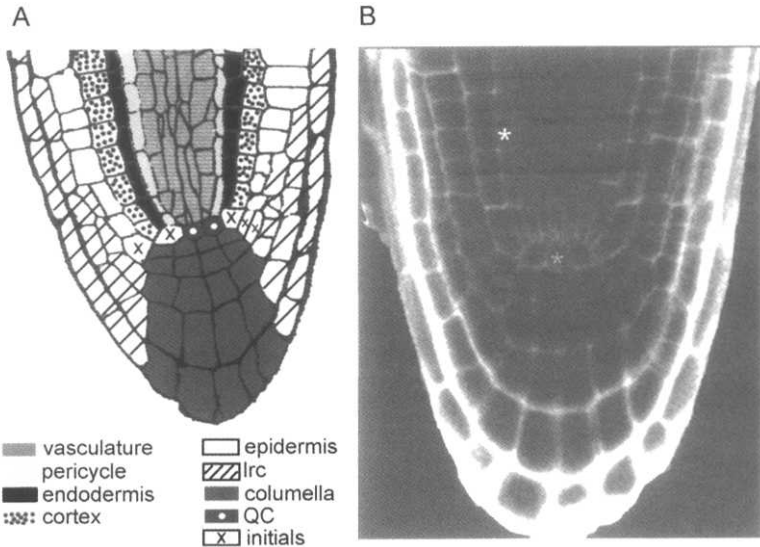


Figure 9.3 Organization of the *Arabidopsis* root meristem. **A:** Schematic representation of the root meristem. The colour code indicates the different tissues of the root. The initials surround the quiescent centre (QC). Initials of lateral root cap (lrc)/epidermis and cortex/endodermis are shown in white. **B:** Confocal image of a 3-day old root tip. The image is a medial longitudinal optical section through the root meristem. The position of the quiescent centre is indicated by a dark grey asterisk. A light grey asterisk shows the endodermis. The root tip has been labelled with propidium iodine, which stains the cell walls.

initial and an upper daughter cell, and the latter then divides asymmetrically in a periclinal orientation. The smaller inner cell will develop as endodermis, while the outer cell will form cortex (Dolan *et al.*, 1993).

In the vascular cylinder, xylem cells form along a single radial axis composed of four to five files of cells that lie very close to the underlying quiescent centre (Mähönen *et al.*, 2000). The outermost cells of this axis form protoxylem cells, while those at the centre differentiate as metaxylem. The protophloem develops in cells positioned at right angles to the xylem axis, and metaphloem develop next to the protophloem cells later in development. The number of periclinal divisions in the procambium is variable, in contrast to the invariant pattern observed in the outer tissues.

Once the radial organization of cells is established in the root, the tangential differentiation of cell types occurs in the epidermis (figure 9.2B). Trichoblasts, which will form root hairs develop in epidermal cells located over two cortical cells when viewed in transverse orientation (figure 9.2C). Cells that contact only one cortical cell when viewed in transverse orientation become atrichoblasts, which will develop as non-root hair bearing cells. Trichoblasts divide faster than atrichoblasts in the meristem and are shorter as a result (Berger *et al.*, 1998a).

9.4 Cell ablation experiments to probe determinants of cell fate of meristematic cells

Cell ablation experiments in biological systems have been instructive in investigating the signals involved in the specification of cell and tissue identity. Cell ablation in roots of *Arabidopsis* is carried out using the laser of a confocal microscope and results in the formation of gaps where ablated cells die. Occasionally, the neighbouring cells move into the gaps created by the dying cells; the developmental response of the cells that move into this new position can be assessed by using different molecular markers of cell identity or morphological criteria. In the following experiments, the role of positional information in the specification of cellular identity is highlighted.

In *Arabidopsis*, epidermal cells differentiate in a position-dependent manner. The role of positional information in the specification of cell identity was examined using laser ablation (Berger *et al.*, 1998b). A row of four to six atrichoblast cells in the middle of the meristematic zone was ablated. Cells from surrounding trichoblast files that invaded the space previously occupied by atrichoblasts did not form a root hair and expressed an atrichoblast specific marker. This indicates that the invading trichoblasts switched identity. Ablation of trichoblast cells was occasionally accompanied by invasion of neighbouring atrichoblasts into the trichoblast position. These invading cells also changed identity, in that they developed root hairs and did not express the atrichoblast marker. Many ablations were accompanied by no invasion of the free space by neighbouring cells. In these cases, the ablation caused no changes in cell identity nearby, indicating that lateral inhibition or a similar cell-to-cell communication process was not active, at least at this stage of epidermal development. Thus, it seems that positional information in the root meristem is a key factor in the specification of cell identity in the epidermis.

In addition, ablation experiments have indicated that the pattern of cell identity does not require living cortical protoplasts, but may be dependent on the walls of the cortical cells. These two observations have led to the suggestion that positional information regulating cell identity in the seedling root epidermis may be located in the cell wall.

An important role for positional information in the specification of the identities of root initials has been shown by an earlier set of laser ablation experiments (van den Berg *et al.*, 1995). The cortical initial undergoes a stereotypical set of divisions that gives rise to the cortex and the endodermis. Laser ablation of the cortical initial resulted in the formation of a gap, into which pericycle cells invaded. These invading cells underwent an asymmetric periclinal division characteristic of the cortical daughter. That is, they changed identity and behaved as cortical cells and not pericycle cells. When the cortical initial was isolated from all contacting daughter cells, by ablation of three adjacent daughter cells, it could still generate a daughter cell but it was not able to divide

asymmetrically. This suggests that signals from the daughter cells are required for cortical initial function. Thus, within an individual cell layer, positional information may signal from the mature regions to the initial. This suggests that initials may not be generators of pattern but instead perpetuate an existing pattern.

Ablation experiments have also been used to define a signalling role in cell differentiation for the central cells of the quiescent centre (van den Berg *et al.*, 1997). When a central cell was ablated, the underlying columella initial differentiated prematurely, as shown by the expression of molecular markers and the accumulation of starch grains, characteristic of differentiated columella cells. This suggests that the central cells repress the differentiation of the columella initial and maintain it in an 'undifferentiated' state.

9.5 Genetic dissection of root development

Cell division and cell specification are generally accepted as being independent from each other in plants. Rather domains of positional information seem to play a major role in determining cell identity. However, evidence is emerging that in certain cases cell division is important in the control of cell specification. The following paragraphs briefly highlight a selection of mutants with defects in meristem formation/maintenance that allow the relationship between cell division and cell identity to be probed (table 9.1). The primary intention is not to be exhaustive, but to illustrate general trends.

9.5.1 Role of auxin in the formation of root meristem in the embryo

Polar auxin transport has been implicated in various developmental processes, such as phototropism, gravitotropism, apical dominance, lateral root formation and vascular tissue patterning. There is an increasing body of evidence indicating that auxin is important during embryonic development, and in particular in the process of embryo-axialization and the formation of the root pole at the base of this axis. A brief description of mutants supporting this role is provided in the following paragraphs.

Plants homozygous for *monopteros* (*mp*) alleles lack both hypocotyl and root (Berleth and Jürgens, 1993). The earliest observable defect in *mp* embryos is an abnormal division of the apical daughter cell of the zygote that gives rise to most of the embryo (Hamann *et al.*, 1999). Discontinuities in the xylem strands form in the mutant, which is phenocopied by treatment of developing leaves with auxin transport inhibitors. Furthermore, *mp* mutants have been shown to be impaired in polar auxin transport (Berleth and Jürgens, 1993; Przemeck *et al.*, 1996). *MP* encodes a protein that is similar to AUXIN RESPONSE FACTOR-1 (ARF1), a transcription factor that binds to auxin-inducible promoters (Hardtke and Berleth, 1998). The gene is initially expressed in broad domains of the embryo and gradually becomes restricted to the vascular system

Table 9.1 *Arabidopsis* genes involved in root development

Gene	Root phenotype
Cell division/pattern formation	
<i>FASS</i>	No regular cell file arrangement
<i>HOBBIT</i>	No root meristem
Maintenance of root meristem	
<i>FASCIATA1,2</i>	Loss of stereotypic cell file arrangement postembryonically
Auxin and root meristem formation	
<i>MONOPTEROS</i>	No root
<i>BODENLOS</i>	No root
<i>AUXIN-RESISTANCE6</i>	No root
<i>GNOM</i>	No basal pole
Radial pattern	
<i>TRN1,2</i>	Lateral root cap cells in place of epidermis, ectopic root hairs
<i>SCARECROW</i>	Single cell layer of ground tissue
<i>SHORT ROOT</i>	Single cell layer of ground tissue
<i>ANTHOCYANINLESS2</i>	Extra cells between cortex and epidermis
<i>WOODEN LEG/CRE1</i>	No metaxylem and phloem
Circumferential pattern	
<i>TRANSPARENT TESTA GLABRA</i>	Ectopic root hairs
<i>GLABRA2</i>	Ectopic root hairs
<i>WEREWOLF</i>	Ectopic root hairs
<i>CAPRICE</i>	Reduced number of root hairs

(Hardtke and Berleth, 1998). This indicates that MP is required for the auxin-mediated development of the root pole in the embryo.

bodenlos (*bdl*) mutants do not form a root meristem in the embryo, whereas roots that develop after germination from the hypocotyl develop normally (Hamann *et al.*, 1999). Some *bdl* seedlings lack not only the root but also the hypocotyl, thus resembling *monopteros*. Defects in the development of the embryo are observed as early as the two-celled stage. Subsequently, the uppermost derivative of the basal daughter cell, destined to become the hypophysis, divides abnormally and fails to generate the central cells of the quiescent centre or root cap. *bdl* seedlings are insensitive to 2,4-dichlorophenoxyacetic acid (2,4-D) and double mutants of *bdl* and *auxin resistance 1* (*axr1*) develop a peg-like structure instead of a root and resemble *monopteros*. These data support the idea that auxin is required for the development of the root during embryogenesis.

auxin-resistance6 (*axr6*) mutants have embryonic phenotypes that are similar to *bdl* and *mp* (Hobbie *et al.*, 2000). Homozygous *axr6* embryos show aberrant cell divisions leading to defects in the suspensor, root and hypocotyl

precursors, and the vasculature, and stop growing soon after germination. Plants heterozygous for *axr6* are viable but have defects in the gravity response, auxin-regulated gene expression, and are auxin-resistant indicating that the mutant *axr6* alleles are dominant. Together, these data support the view that auxin is required for the development of the root pole during embryogenesis.

Indole acetic acid (IAA) is an auxin that moves from the apex of the plant, where it is synthesized, to the root. It has been proposed that auxin is taken up into cells via carriers, such as AUX1, and transported out of cells by proteins of the PIN family. PIN1 is a putative efflux carrier, localized in the basal part of vascular cells in the axis of *Arabidopsis* plants, and is predicted to be the site of auxin efflux (Gälweiler *et al.*, 1998). The distribution of PIN1 in embryos has implicated this protein in the development of the basal pole, the site of root meristem development (Steinmann *et al.*, 1999). Early in embryogenesis, PIN1 protein is distributed in a non-polar fashion in cells. As embryogenesis progresses, PIN1 assumes an asymmetric distribution. By the globular stage of embryogenesis, the four inner cells at the base of the embryo proper localize PIN1 to the plasma membrane on the basal end of the cell facing the future root tip. This polar localization of PIN1 at the basal faces of cells in the embryo requires GNOM, a G-protein that has been implicated in vesicle targeting in cells. Mutants lacking *GN* function are also unable to form the basal root pole (Mayer *et al.*, 1993). Together, these data suggest that *GN*-mediated localization of PIN1 is required for the formation of the basal pole where the future root meristem develops.

9.5.2 Maintenance of the root meristem organization

We can formally separate root development into two distinct processes. The earliest phase involves the generation of the root meristem and its associated tissue organization in the embryo. Subsequently, this pattern is actively maintained in the roots of seedlings. The identification of mutants that undergo normal embryonic development, but have abnormal post-germination root development, has defined genes required for the maintenance, as distinct from the generation, of the root meristem. For example, *FASCIATA* (*fas*) genes have been shown to play a role in meristem maintenance after germination.

Root meristem formation is normal during embryogenesis in *fas1* and *fas2* mutants, but the wild-type organization of the meristem is not maintained during subsequent growth (Kaya *et al.*, 2001). At 6 days, the stereotypic arrangement of the central cells and initials is lost, making central cells difficult to distinguish from surrounding initials. Marker gene expression in the mutant meristem is altered in a stochastic manner, showing an expression or loss of expression within a single root tip, thus strengthening the maintenance role of these genes. The *FAS* genes have been cloned and encode subunits of the chromatin assembly factor-1 (CAF-1). It has been postulated that *FAS* genes facilitate the stable

maintenance of gene expression in the apical meristems ensuring the constant propagation of epigenetic states of chromosomes.

9.5.3 *Is there a correlation between cell division and cell differentiation?*

The ability to undergo continuous cell division is one of the defining characteristics of a meristem. The simple pattern of cell divisions and small cell numbers have allowed the characterization of mutants with defects in the cellular organization of the root meristem or the onset of post-embryonic cell division. *fass* (*fs*) mutants have abnormal cell divisions, but patterning of cells and tissues appears normal indicating that cell division and cell specification are separate processes. On the other hand, *hobbit* (*hbt*) mutants exhibit cell division defects, but also have defects in the specification of cells in the root, suggesting that there may be a connection between cell division and cell specification. In the following paragraphs the phenotypes of these mutants are described in more detail.

The orientations of cell divisions are defective in *fs* mutants. *fs* seedlings are compressed in their apical-basal axis and enlarged circumferentially (Torres-Ruiz and Jürgens, 1994). The pattern of cell divisions and expansion are defective from the zygotic stage. Defective alignment of microtubules may be responsible for the *fs* phenotype, suggesting that FS directly or indirectly regulates microtubule organization (Traas *et al.*, 1995). Even though cell division defects persist through embryogenesis, all the elements of body pattern are differentiated in *fs* mutant seedlings. The root lacks the regular arrangement of files of cells found in the wild-type, but it does form a stele and an epidermis that makes hairs. This observation again suggests that specification of cell identity and cell differentiation are not dependent on cell division per se. Cell division is defective, but cell specification is almost normal.

hbt mutants were identified in screens for mutants with defective embryonic root development (Willemsen *et al.*, 1998). The planes of cell divisions are defective in mutant embryos from the quadrant stage onwards, indicating that HBT function is required for the orientation of cell divisions during embryogenesis. By the time embryos reach the torpedo-stage, the periclinal divisions that give rise to the lateral root cap and epidermis are either misaligned or absent, and cell numbers in the cortex and endodermis are reduced compared to wild-type. Plants homozygous for strong *hbt* alleles develop into seedlings with no recognizable quiescent centre or differentiated columella root cap cells. Not only are the cells in these regions disorganized in *hbt* mutants, but they also fail to express cell-specific marker genes. This indicates that, in addition to defects in cell division, there are also defects in the specification of cell identities in the *hbt* mutants. Thus, at certain levels there seems to be a connection between cell division and the specification of cell identities in the root. This contrasts with the apparent independence of these processes in *fs* mutants. Future research will be

instructive in elucidating how cell division and differentiation are interconnected during root development.

9.5.4 The development of the radial organization of cells in the root

The radial organization of the outer cell layers of the root is progressively elaborated during embryogenesis and maintained during the postembryonic growth of the seedling. Genetic dissection of the process of root formation has led to the identification of genes required for development of the radial organization of cell layers. For example, *SHORTROOT* and *SCARECROW* are required for the formation of the endodermis and cortex from a single cell layer during embryogenesis, since *shr* and *scr* mutants have a single layer instead of distinct endodermal and cortical cell layers. The radial pattern in wild-type roots is subsequently maintained after germination by regular divisions of the initial cells. The following sections describe a number of mutants impaired in the radial organization of cell types in the root, and emphasize the role of the corresponding genes in the postembryonic development of the root.

In the growing root, the protodermal initial divides periclinally to form an outer lateral root cap cell and an inner epidermal cell. The divisions of the initial are occasionally defective in *tornado1* (*trn1*) and *trn2* mutants, indicating that *TRN1* and *TRN2* are required for this division to occur (Cnops *et al.*, 2000). This defective division can cause a disruption in the cellular organization of the epidermis and lateral root cap cells, and occasionally results in the formation of an exceptionally large protodermal initial. Furthermore, in young seedlings (3–5 days old) a subset of the epidermal cells develop as lateral root cap cells. As the root ages, the number of these mis-specified epidermal cells increases. This indicates that *TRN1* and *TRN2* are involved either in the positive regulation of epidermal fate, or the negative regulation of lateral root cap fate in cells of the epidermis. *trn* mutant embryos are morphologically normal, indicating that *TRN1* and *TRN2* are required only for the maintenance of the radial pattern in the seedling and not for its development in the embryo.

SCARECROW (*SCR*) and *SHORT-ROOT* (*SHR*) are necessary for the periclinal division of the cortex/endodermis initial daughter cell in growing roots (Scheres *et al.*, 1995). Loss-of-function mutations in these genes result in the formation of a single ground tissue layer instead of the two distinct files of cortex and endodermis. In *scr* mutants, this layer has attributes of both cortex and endodermis, indicating that *SCR* is important for cell division (Scheres *et al.*, 1995; Di Laurenzio *et al.*, 1996). *shr* mutants have a single cortical layer and no endodermis, suggesting that *SHR* is required for both cell division and differentiation (Helariutta *et al.*, 2000). The role of *SHR* in cell division is further supported by the fact that ectopic expression of *SHR* results in the occurrence of extra periclinal cell divisions in the root meristem forming supernumerary cell layers. *shr fs* double mutants result in an increase of cell layers, a characteristic of *fs* single mutants. However, no endodermis developed in

these double mutants, indicating that *SHR* is not only required for division of the cortical daughter but also for the differentiation of the endodermis. *scr fs* double mutants also have extra layers, but form an endodermis (Scheres *et al.*, 1995). This further supports the view that *SHR* is important for cell division and differentiation of the endodermis, while *SCR* is only required for cell division of the initial.

SCR and *SHR* are members of the GRAS family of transcription factors (Di Laurenzio *et al.*, 1996; Helariutta *et al.*, 2000). *SCR* is expressed in the quiescent centre, the endodermis/cortex initial and throughout the endodermis. This suggests that *SCR* is required in the cells in which it is expressed. *SHR*, on the other hand, is expressed in the stele. The *SHR* expression pattern indicates that *SHR* acts in a non-cell-autonomous manner (Helariutta *et al.*, 2000). Genetic analysis has shown that *SCR* and *SHR* act on a common pathway, since double mutants exhibit an epistatic interaction of *shr* on *scr*. The decreased amount of *SCR* mRNA in *shr* mutants suggests that *SHR* is required for the transcription of *SCR*.

The *ANTHOCYANINLESS2* (*ANL2*) gene codes for a homeodomain protein affecting anthocyanin distribution and is required for the organization of cells in the root (Kubo *et al.*, 1999). Extra cells, which resemble cortical cells, form between the cortical and epidermal layers cells in *anl2* mutants. It is likely that these extra files of cells arise from divisions in the meristem, although it is not known if these divisions occur in the epidermis or cortex. Its precise role in the radial organization of the root remains to be determined.

The *WOODEN LEG* (*WOL*) gene is required for the development of the cells in the vasculature of the stele. *wol* mutants have fewer initial cells in the stele, and its vascular system comprises only protoxylem. Metaxylem and phloem are absent (Mähönen *et al.*, 2000). *WOL* is required for a set of divisions that occur first during the late stages of embryogenesis, and is necessary to propagate this pattern of cell division in the root meristem after germination. The role of *WOL* in metaxylem and phloem differentiation was analysed using double mutants of *wol* and *fs* (Scheres *et al.*, 1995; Mähönen *et al.*, 2000). The number of cells in the stele is increased in *wol fs* double mutants and both phloem and metaxylem develop. This indicates that *WOL* is required primarily to control cell number in the stele, and its effect on phloem and metaxylem differentiation is likely to be secondary, resulting from decreased cell number in the mutant stele.

WOL is a member of a family of two component regulators that are thought to act as receptors for extracellular signals in plants. It is expressed in procambial cells of the embryonic and primary root. It has, therefore, been postulated that *WOL* is a receptor that controls asymmetric cell divisions of the vascular initials through a specific signal transduction pathway that involves a phosphotransfer reaction characteristic of the two component receptors. *WOL* is allelic to *CYTOKININ RESPONSE1* (*CRE1*), which has been identified as a cytokinin receptor (Inoue *et al.*, 2001). By extension, this suggests that cytokinin may have a role in controlling the number of cells in the stele of the root.

9.5.5 *Specification of cell identity in the epidermis*

The circumferential pattern of alternating trichoblasts and atrichoblasts in the root epidermis develops during embryogenesis and is propagated in the postembryonic seedling root. Two broad classes of mutants have been instructive in elucidating the molecular mechanism underpinning the development of this alternating pattern. The first are mutants with ectopic root hairs, where hair cells develop in the position of non-hair cells, and the second are mutants lacking or having a reduced number of root hairs.

Plants homozygous for loss of function mutations in three genes, *TRANSPARENT TESTA GLABRA* (*TTG*), *GLABRA2* (*GL2*) and *WEREWOLF* (*WER*), develop root hairs in all cells of the epidermis. This indicates that the genes are negative regulators of hair cell identity. *TTG* encodes a WD-40 repeat protein (Walker *et al.*, 1999) that is thought to mediate protein interactions in protein complexes, including transcription complexes. *WER* encodes a MYB-related protein that is expressed in atrichoblasts (Lee and Schiefelbein, 1999), suggesting that its activity represses hair identity in these cells. *GL2* encodes a homeodomain protein that is expressed in atrichoblasts, and is regulated by *WER* and *TTG* (Di Christina *et al.*, 1996).

Plants homozygous for the loss of function *cpc-1* mutation develop fewer root hairs compared to wild-type, suggesting that *CPC* acts as a positive regulator of root hair formation. *CPC* encodes a protein with a single MYB-domain, which lacks an activation domain required for transcription (Wada *et al.*, 1997). This suggests that *CPC* may act as a repressor of transcription by binding to promoter targets and blocking the activation of transcription. On the basis of these studies, a model has been proposed in which the relative levels of *CPC* and *WER* determine the fate of a cell. High cellular levels of *CPC* relative to *WER* are predicted to result in the development of a trichoblast (hair). On the other hand, high levels of *WER* relative to *CPC* in an epidermal cell results in the formation of an atrichoblast.

9.5.6 *TTG, WER and CPC regulate cell division*

It has been suggested that the cell cycle of trichoblasts is shorter than atrichoblasts and that this difference can account for the fact that trichoblasts are shorter than atrichoblasts. This difference in cell division between the two cell types is regulated by *WER*, *TTG* and *CPC*. All epidermal cells are short in *wer* and *ttg* mutants (Berger *et al.*, 1998a; Lee and Schiefelbein, 1999), suggesting that *WER* and *TTG* are required for the 'long' cell cycle. Epidermal cells of *cpc* mutants, on the other hand, are all of the long (atrichoblast) type indicating that *CPC* is required for the formation of the short trichoblast cells. While the root hair phenotype of *gl2* mutants resembles *wer* and *ttg* mutants, cell division rates are different in the two epidermal positions in *gl2* mutant roots. This suggests

that 'short cell size' is not a prerequisite for hair development. Thus, cell division parameters and cell identity can be uncoupled during the development of the epidermis.

9.6 Perspectives

The isolation of mutants with defects in root development, and the cloning of the respective wild-type genes is providing insights into the molecular mechanisms underpinning the formation of the root meristem in the embryo and its maintenance in the seedling root. It is anticipated that continued genetic analysis will identify signalling pathways that regulate the activity of transcription factors that have already been identified, such as *SHR* and *SCR*. Furthermore, we expect that genetic analysis will identify the overlap between different aspects of root development; for example, the connection between radial and circumferential patterning in the epidermis. Ideally, this long-term genetic analysis of root development will provide tools with which the environmental control of root development can be studied, showing mechanistically how nutrient availability can modify the development of whole root systems.

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Index

- ABC model 113, 120, 123, 124
aberrant leaf and flower (alf) 150
ABERRANT PHYLOTAXY (ABPHYL) 185, 187, 188
 ablation 148
 abscisic acid (ABA) 95, 102, 103, 265, 266
ABSCISIC ACID INSENSITIVE (ABI1) 95, 102
Acer 175, 239
 ADP-ribosylation 62
AFILA (AF) 241, 242
AGAMOUS (AG) 97, 106, 112-114, 120, 121, 123-126
AGAMOUS -LIKE (AGL)
 AGL2 114, 124
 AGL4 114, 124
 AGL8 106, 109
 AGL9 109, 114, 124
 AGL20 97, 105, 128
AGL20/SOC1 95
Ailanthus 176
AINTEGUMENTA (ANT) 37, 38, 42, 70, 72, 106, 121, 146, 185
alfalfa 266
ALTERED MERISTEM PROGRAM (AMP)-PROGRAMME (AMP0-AMP) 34, 162, 185
Anagallis 175
 anaphase 255, 260
ANGUSTIFOLIA (AN) 233
 anneau initial 178, 217
ANTHOCYANINLESS2 (ANL2) 289
 anticlinal/periclinal border 6
Antirrhinum 20, 39, 46, 89, 90, 105, 107, 110-113, 116, 118, 123, 128, 146, 150, 151, 163, 175, 221, 242, 258, 270, 271
APETALA (AP)
 AP1 97, 100, 106-114, 116-121, 123, 124, 126, 128
 AP2 70, 97, 106, 115, 121, 123-127
 AP3 78, 97, 106, 114, 120, 121, 123, 124, 125
 apical dominance 161
 apical initial 19, 217
 apical-basal axis 60, 63
 apical-basal polarity 61
 apoplastic 47
 apple 21
ARABIDOPSIS MERISTEM LAYER1 (ATML1) 45
ARABIDOPSIS SHAGGY-related protein KINASE (SKP1)-LIKE PROTEIN (ASK)
 (*ASK1*) 78, 97, 106, 122, 125
 (*ASK2*) 78
Arabidopsis thaliana Lipid Transfer Protein1 (AtLTP1) 62
Araucaria 173
arborescent (arb) 150
 arginine decarboxylase 43
ARGONAUTE1 (AGO1) 27, 28, 77, 224
ARRESTED DEVELOPMENT (ADD1) 185, 186
Asclepias 145
 ascorbic acid oxidase (AAO) 5
 ash 235
ASYMMETRIC LEAVES 1 (AS1) 37, 38, 70, 71, 72, 78, 79, 81, 227
 atrichoblasts 282, 283, 290
AUGUSTIFOLIA (AN) 233
 auricle 225
 auxin 5, 33, 43, 122, 158, 160, 161, 164, 172, 186, 187, 189, 193-200, 202-204, 263, 264, 271, 284, 286
 canalization 198
 efflux carrier 42, 62, 186, 194
 polar transport 42, 72, 172, 190, 194-196, 199
 transport 193, 197, 284
 transport inhibitor 194
 2,3,5-triiodobenzoic acid (TIBA) 192
 N-1-naphthylphthalamicNPA 193
 response mutants 161
AUXIN RESISTANCE PROTEIN (AXR) 160, 263
 AXR1 263
 AXR3 156
 AXR6 285, 286

- AUXIN RESPONSE FACTOR (ARF) 62, 63
 ARF1 284
 ARF5 67
 axial patterning 220
 axialization 67
 axillary meristem
 initiation 157
 ontogeny 152, 155
Azolla 4
- barley 24, 92, 164, 165
 basal cell 60
Begonia hispida 164
 bijugate 193
 bilateral pattern 72
 birch 47
 blade 225
 blastozone 235
BLEC 7
 blue-light receptor 95, 100
BODENLOS (BDL) 285
BRACTEA (BRA) 148
branched silkless1 110
 branching types 142
Brassica 100
Brassica juncea 62, 72
 brassinolide 261
 brassinosteroid 233, 265
 brefeldin A (BFA) 62, 63
 brown algae 2
 bryophytes 2, 21
 bud dormancy 162
 bZIP family 189
- C2H2-type zinc finger 114
 cactus 173
Caenorhabditis 77
 cambial zone 5
 CAMV (35S) promoter 100, 104, 105, 109,
 111, 112, 114, 118, 125
 canalization hypothesis 197
 capitulum 38, 90, 91
Capparaceae 188
Capsella 60
CARPEL FACTORY (CAF) 106, 114, 115,
 156
CAULIFLOWER (CAL) 97, 106, 107, 109,
 111, 112, 128
CCA1 95, 97, 99, 100
 CCAATT box 65
 CDK activating kinases (CAKs) 256, 258,
 262
- CDK inhibitor proteins (CKI) 259, 266
Cki1At 259, 265
Cki2At 259
Cki3At 259
- cell
 ablation 283
 commitment 20
 cycle 5, 20, 28, 200, 201, 254
 cycle genes 200
 division 218, 284, 287
 expansion 232
 fate 18
 wall microfibrils 204
- cell division cycle (*cdc*) genes
cdc2 256, 258, 263, 266, 267
 CDC25 259, 268
 CDC25-like 264, 266
 CDC2At 257, 263
 CDC2bAt 257, 258
- cdc2* kinase 231
 cellulose microfibrils 39, 182, 203
CEN-like genes (CET) 110, 151
 central domain 36
 central mother cells 19
 central zone (cz) 7, 8, 19, 58, 118, 119, 178,
 179, 181, 217, 270, 272
CENTRORADIALIS (CEN) 110, 112, 116,
 127, 151
Ceratopteris 21
 chalaza 59
 chalaza-micropyle 61
Chamaedorea 145
Chisocheton 234
- CHROMATIN ASSEMBLY FACTOR I
 (CAF1) 32, 81
Chrysalidocarpus 164
Chrysanthemum 192
 Cip/KIP 259
 circadian clock 95, 98, 99, 100
 CK2 protein kinase 99
 CKS proteins 259
CLAVATA (CLV) 8, 29, 32, 72, 79, 80, 82,
 115, 183, 246, 271, 272
CLV1 8, 29-32, 72, 79, 80, 106, 116, 119-
 121, 179, 180, 181, 183-185, 187, 272
CLV2 29-32, 72, 106, 119, 121, 272
CLV3 29-33, 46, 47, 72, 79, 80, 106, 116,
 119-121, 179, 180, 183-185, 187, 272
- CAPRICE (CPC)* 290
 clonal analyses 217
cochleata 243
Coleus 175

- columella initial 284
 columella root cap 67
 compound leaf 234
 compound leaf development 235
Coniferophyta 145
CONSTANS (CO) 94, 95, 97, 100-105, 113, 117
 contact parastichies 173
 cork cambial meristem (CCM) 2
Cornus 176
 corpus 7, 17, 18, 38, 47, 58, 177, 182
 cortex 282, 288
 cortical initial 283
 cortical microtubules 204, 255
 corymbs 90, 91
 cotyledon 25, 59, 60
 cotyledon formation 70
CRABS CLAW (CRC) 224
 cranberry 20
 crucifers 195
CRYPTOCHROME (CRY) 95, 97, 98, 100
CULLIN 122
CUP-SHAPED COTYLEDONS (CUC) 42, 43, 69, 70, 72, 73, 75, 76, 80, 81, 82, 184-186, 187, 271
 CUC1 69, 186
 CUC2 41, 186
CURLY LEAF (CLF) 97, 106, 126
Cycadophyta 145
 cycads 16
 cyclin 256, 257
 A-type cyclins 257, 260, 266, 267
 B-type cyclins 258, 260, 266, 267, 269
 D-type cyclins 20, 33, 34, 37, 39, 46, 258-262, 264-266, 269-271, 273
 H-type cyclins 258
 cyclin/CDK complexes 259, 261, 262
 cyclin-dependent-kinase (CDK) 257-259, 260, 264, 266, 267, 269, 273
 CDK-*a* 256-260, 262, 263, 265-270
 CDK-*b* 256-258, 265-270
 CDK-*c* 256
 CDK-*d* 256
 CDK-*e* 256
 p34CDKA 262
 CDK inhibitor (CKI) 259, 268
 cymes 148
CYP78A5 7, 36
 cytochrome P450 36, 232
 cytokinesis 256
 cytokinin 33, 34, 105, 159, 162, 164, 193, 261, 263, 264, 265
 cytokinin-autotrophic tobacco cell suspension line (BY-2) 263
CYTOKININ RESPONSE1 (CRE1) 289

Dactylis glomerata 269
 daisy 90
Daphne pseudomezereum 17
Datura stramonium 7, 18
 D-Box 259
decreased apical dominance (Dad) 162
 decussate 172, 174, 175, 176, 182, 189, 193, 199
 deepwater rice 262
DE-ETIOLATED2 (DET2) 95
 DEF 124
DEFECTIVE EMBRYO AND MERISTEMS (DEM) 28, 184
 detached meristems 152, 153, 155, 165
 determinacy 233
 DEXH/DEAD-box helicase 115
Dianthus chinensis 90
 Dichasium inflorescence 90, 91
 dichotomous 142, 145
diphtheria toxin A chain 148
 disrupted (dip) 184
 distichous 172, 174, 182, 199
 divergence angle 174
 DNA
 helicase 255
 methyltransferase gene (*MET1*) 101
 polymerase 255
 dorsiventrality 220
 asymmetry 221
 patterning 229
Downingia 60, 82
Drosophila 77
Dryopteris 8, 173, 191, 192
Dryopteris dilatata 6
 dUTPase 43

 E2F 261, 262, 265, 266
 E2F-binding sites 262
EARLY FLOWERING (ELF) 96
 ELF1 95
 ELF2 95
 ELF3 95, 97-100
EARLY FLOWERING IN FWA MUTANT (EFS) 95, 101
EARLY FLOWERING IN SHORT DAYS (ESD)
 ESD1 95
 ESD2 95

- ESD3* 95
ESD4 95
EARLY FLOWERING1 (EAF1) 95, 102
 ectopic organogenesis 22, 24, 34, 196, 202, 237
 egg 59
eIF2C 77
 ELK domain 22
 embryo 17
 proper 59, 60
 sac 61
EMBRYO DEFECTIVE30 (EMBO30) 61-63
 embryogenesis 26
EMBRYONIC FLOWER (EMF) 95-97
 Enation 213
 endodermis 281, 288
ENHANCED RESPONSE TO ABA (ERA) 119, 184, 185
Ephedra 18
 epidermis, specification of cell identity 290
Epilobium 176, 193
 epiphyllous structure 164
Equisetum 3, 145
ETHYLENE INSENSITIVE (EIN2) 95
 ethylene receptor (ETRI) 95
ETTIN (ETT) 106, 122, 185, 189
Euphorbia esula 4
 expansins 38, 201, 202, 203, 218
extra petals (exp) 148
 extracellular leucine-rich repeats (LRRs) 30

falsiflora (fa) 108, 150, 243
FASCIATA (FAS) 81, 183, 286
 FAS1 29, 32, 81, 184
 FAS2 29, 32, 81, 184
fasciated 28, 29
FASS (FS) 39, 66, 287
F-box 78, 98, 116, 122
F-box protein 107, 122
fbp2 114
FD 94, 95
FE 94, 95
 ferns 233
FHA 94, 98
FHA/CYR2 95, 103
fhy1 96
 Fibonacci angle 173
FIDDLEHEAD (FDH) 44
 field model 192
fil 116, 224
 fil ap1 double mutant 116
 fil lfy double mutant 117

FILAMENTOUS FLOWER (FIL) 17, 71, 106, 116, 122
 file zone 7
FIMBRIATA (FIM) 115, 116, 122
 first available space model 191, 192
Flagellaria 145
FLAVIN-BINDING, KELCH REPORT F-BOX (FKFI) 95, 97, 98, 99
FLORICAULA (FLO) 107-110, 112-114, 127, 185, 188, 242
FLOWERING LOCUS C (FLC) 95, 97, 104, 105, 127
FLOWERING LOCUS D (FLD) 94, 95, 97, 100, 101
FLOWERING PROMOTER FACTOR1 (FPF1) 95, 102
FLOWERING TIME (FT) 94, 95, 97, 103-105, 117, 118
FLOWERING TIME CONTROL PROTEIN A (FCA) 40, 94, 95, 97, 100, 102-105, 117-119
FOREVER YOUNG (FEY) 183-186
 forkhead-associated (FHA) domain 31
 founder cell 35
FPA 94, 95, 97, 100, 101, 104
FRIGIDA (FRI) 95, 97, 104
FRUITFUL (FUL) 106, 109
Fucus 2, 63
FULLY FASCIATED (FUF) 183, 184
FUSCA3 64, 65
FUSION OF FLORAL ORGANS (FFO)
 FFO1 106, 116
 FFO2 106, 116
 FFO3 106, 116
 fuzzy morphology 245
FVE 94, 95, 97, 100, 102, 104
FWA 95, 97, 102, 103, 104, 105, 117
fva lfy double mutant 118
FY 94, 95, 97, 100

 G0-G1 transition 260
 G1 phase 97, 254, 255, 257, 260, 261, 262, 264, 266, 269
 G1/S arrest 264
 G1/S transition 254, 257, 260, 262, 263, 264, 273
 G2 phase 254, 255, 257, 260, 264, 265, 266, 267
 G2/M transition 254, 257, 258, 260, 263, 264, 265, 270
GAI 95, 97, 102
 generative spiral 173

- germ cells 18
- gibberellic acid (GA) 96, 103, 105, 113, 115, 117, 127, 156, 157, 193, 244, 262, 263
 - GA 20 oxidase 239
- GIBBERELIC ACID INSENSITIVE**
 - (*GAI*) 95, 97, 102, 156
- GIGANTEA* (*GI*) 94, 95, 99, 100, 102, 104
- Ginkgo* 19, 108, 173
- Ginkophyta* 145
- GLABRA2* (*GL2*) 290
- global patterning 231
- GLOBOSA* (*GLO*) 124
- β -glucuronidase (*GUS*) 230
- glumes 93
- GNARLEY 1* 225
- Gnetum* 18
- GNOM* (*GN*) 61-63, 82, 246, 286
- GRAS 289
- groove meristem 165
- guanine nucleotide exchange factors
 - (GEFs) 62, 63
- Guarea* 234
- HAP3 65
- Hdl* 100
- Helianthus* 19, 176
- Heracleum* 19
- heterochronic 242
- Hidrocharis morsus-ranae* 153
- Hieracium floribundum* 153
- HOBBIT* (*HBT*) 67, 68, 287
- homeobox-leucine zipper (HD-ZipIV) 44, 45
- homeodomain 23, 45, 96, 100, 104, 106, 107
 - protein 95, 118
 - transcription factors 22
- homeotic transformations 239
- Hooded* 24, 164, 165
- HvKnox3* 24
- 3-hydroxy-3-methylglutaryl (HMG)-CoA
 - reductase 263
- Hypanthodium* 90, 91
- Hypericum* 175
- hypocotyl* (*HY*) 25, 95
 - HY1* 95, 115
 - HY2* 96
 - HY4* 98
 - HY8* 96
- ID1* 101
- incipient leaf formation 191-193
- indeterminacy 236
- indeterminate growth 237
- indeterminate spikelet 1* (*IDS1*) 115
- indeterminate1* (*id1*) 101
- indole-3-acetic acid (IAA) 43
- indole-3-acetic acid lysine synthetase*
 - (*iaaL*) 161
- indoleacetamide hydrolase* (*iaaH*) 161
- interfascicular fiberless1 (*ifl 1*) 184
- intermediate forms 244
- Iris tectorium* 91
- irradiation 21
- isopentenyl transferase* (*ipt*) 162, 164
- isopentyl transferase 33
- Japanese honeysuckle 91
- KNAT1* 26, 34, 37, 71, 163
- KNAT2* 71
- KNOTTED1* (*KN1*) 22-24, 36, 37, 47, 163, 165, 184, 218-220, 225, 228
 - Oskn3* 40
 - Oskn4* 237
 - Oskn5* 237
- KNOTTED-LIKE HOMOEBOX* (*KNOX*) 21, 22, 23, 24, 26, 35, 36, 40, 46, 70, 71, 79
 - Knox3* 23, 40, 165
 - Knox4* 23, 40
- L₁* layer 17, 18, 29, 31, 33, 43-45, 47, 177, 203, 270
- L₂* layer 17, 29, 31, 33, 178, 270
- L₃* layer 17, 18, 27, 29, 178, 179, 181
- L38_46*
- Lamiaceae* 91, 175, 176
- LAMINA1* (*LAMI*) 228, 229
- Laminaria* 2
- Lancelolata* 238
- laser ablation 13
- LATE ELONGATID HYPOCOTYL* (*LHY*) 96, 97, 99, 100
- lateral domain 229
- lateral patterning 227
- lateral suppressor* (*ls*) 156, 158, 184
- LD* 94, 96, 97, 100, 101, 104, 117
- leaf initiation 220
- leaf primordia 17
- leaf shape mutants 238
- leafbladeless* (*lbl*) 223
- leaflets 234
- LEAFY COTYLEDON1* (*LEC1*) 64, 65
- LEAFY* (*LFY*) 78, 97, 100, 106-114, 116-121, 123, 125, 127, 146, 185, 188, 195, 195243

- LeExp18* 38, 202, 203
 lemma 93
LEUNIG (LUG) 97, 106, 122, 125, 126
 ligule 225
LIGULESS 3 225
LIGULESS 4 225, 226
Ligustrum 4
Liliaceae 175
 lipid transfer protein (LTP) 43, 179
 lodicule 93
Lonicera japonica 91
LOV KELCH PROTEIN (LKP1) 98
 lovastatin 263, 264
 LRR 30, 32
 lupin 173, 191
Lycopersicon esculentum 28
Lycophyta 143
Lycopods 145

 MADS-box 95, 96, 105, 106, 109, 110, 114, 124
 MADS-domain 104, 109, 114, 124
 magnolia 20, 90
Magnoliophyta 145
 maize 18, 36, 44, 89, 90, 93, 110, 115, 259, 262
Malaxis paludosa 164
 Mammillaria 145
 MAP kinase 103, 110, 118
 maternal effect mutants 61
 maturation schedule 226
 MCM proteins 255
 mechanical forces 9
 megaphyll 213
Mentha 175
 meristem 6
 - adventitious 25
 - apical 2
 - autonomy 10
 - boundaries 6
 - cork cambial 2
 - definition of 2
 - fractionation 235
 - inflorescence 42
 - intercalary 2, 231
 - lateral 2
 - maintenance of 18, 21, 23
 - marginal 229, 230, 235
 - primary 2
 - rib 19
 - vascular cambium 2
 méristème d'attente 3, 178
 merophytes 3, 6
 mesodermal tissue 18
 metaphase 255, 260
 metaphloem 282
 metaxylem 282
 mevalonic acid 263
MGOUN (MGO)
 - MGO1* 35, 106, 119, 120, 184
 - MGO2* 35, 106, 119, 120, 184
 microphyll 213
 micropyle 59
 MIENOX domain 22
 mitogen-activated protein kinase (MAPK) 31
 mitotic indices 218
 mitotic spindle 255, 260
Monophyllaea 167
Monophyllaea horsfieldii 165, 166
 monopodial 142, 145, 146
 monopodial branching 158
MONOPTEROS (MP) 66, 67, 181, 185, 186, 199, 284, 285
 morphology 244
 mosaic analysis 226
 M-phase 254, 255, 257, 258, 260, 263, 266, 267
Myb 37, 95, 96, 99, 219, 222

 NAC domain 41, 75, 82
NAM 42, 76, 82, 186, 187
 N-1-naphthylphthalamic acid (NPA) 190
NARROW SHEATH (NS) 220, 228
NEEDLY 108
 negative field model 191
Nerium oleander 175
Neurospora 77
Nicotiana sylvestris 228, 264
NICOTIANA TABACUM HOMEBOX (NTH)
 - NTH9* 46
 - NTH15* 45, 239
 - NTH20* 45, 46*NO APICAL MERISTEM (NAM)* 41, 42, 76, 82, 184-187
NPH1 98
nuphar 233
Nypa 145

 oat 93
Okra 229
 organizational centers 10
ORYZA SATIVA HOMEBOX (OSH)
 - OSH1* 24, 35, 77, 78
 - OSH15* 40, 41

- OSH43* 24
- Osmunda* 3, 173
- Ostrich fern 152
- ovule 59
- Palea 93
- panicle 93
- Papaver* 60
- PAS/LOV domain 98
- pasticcino1-3 (*pas1-3*) 184
- pea 162, 239
- pentamerous phyllotaxis 175
- perianth 93
- PERIANTHIA (PAN)* 106, 121, 123, 185, 188, 189
- periclinal chimeras 7, 17, 30
- pericycle 281, 282
- perigon leaves 176
- peripheral zone (PZ) 7, 8, 19, 20, 38, 58, 118, 119, 177-179, 181, 196, 217, 270
- PETAL LOSS (PTL)* 106, 122
- Petunia* 41, 76, 89, 114, 146, 148, 150, 162
- PHABULOSA (PHAB)* 26, 163, 223, 247
- PHANTASTICA (PHAN)* 36-38, 70, 163, 219, 221-224, 227, 228, 247
- Phaselous* 233
- phabulosa-1d (phb-1d)* 163, 223
- phellogen 2
- phragmoplast 256, 260
- phyllotaxy 40, 92, 172, 174, 177, 181-183, 187-190, 195, 196, 204
- PHYTOCHROME (PHY)* 96, 98, 100
- PHYA* 96-98
- PHYB* 97-99, 117
- PHYD* 96, 97
- PHYE* 96-98
- pine 108
- PIN-FORMED1 (PINI)* 42, 43, 62, 63, 180, 181, 185-187, 189, 193-195, 198, 199, 286
- transporter 43
- PINHEAD/ZWILLE (PNH/ZLL)* 76, 77, 81, 155, 156, 165-167, 184, 224
- pinnae 234
- identity 242
- PINOID (PID)* 71, 180, 181, 185, 186, 189
- Pinus* 108, 173
- PISTILATA (PI)* 106, 114, 120, 121, 123, 124, 125
- Pisum* 200
- Pisum sativum* 174
- plasmodesmata 24, 47
- plastochron 39, 173, 193, 216
- ratio 175, 176
- PLENA (PLE)* 113
- polycomb group proteins 122, 126
- polyphenoloxidase 43
- Polytrichum* 3
- poplar 173
- positional information 283
- post-transcriptional gene silencing 77
- potato 8, 174
- potato leaf* 238
- pre-prophase 267
- primary root organization 280
- probability mapping 21
- procambium 282
- progenitor cells 3
- Progymnospermophyta* 143, 145
- prophase 255, 260
- proteasome 259
- protein kinases 256
- protein phosphatase (KAPP) 31, 95
- protein-protein interactions 22, 99, 102
- protoderm 44
- PROTODERMAL FACTOR1 (PDF1)* 45
- protodermal initial 288
- protophloem 282
- provascular calls 12
- proximal/distal 223, 231
- patternning 225
- PsDRM1* 162
- Pseudomonas savastanoi* 161
- pseudo-monopodial 143
- pseudo-sympodial 143
- Psilophyta* 143
- Psilophyton crenulatum* 143
- Psilotidae* 143
- Psilotum* 145
- Pteridophyta* 143, 145
- Pteridospermophyta* 145
- PTGS 77
- QDE-2 77
- quadrimerous phyllotaxis 175
- quantitative trait locus (QTL) 100
- quelling 77
- quiescent center (QC) 4, 5, 281, 282
- raceme 90, 148
- rachis 234
- radial domains 19
- radial pattern 72, 288
- Raf kinase 103

- ramosa1* 109
ramosa2 110
Ramosus (rms) 162
 Ras 261
rasperry 64
 RDE-1 77
 red light receptor 96
 replication complex 255
REPRESSOR OF GALI-3 (RGA) 96, 156
 retinoblastoma (rb) 266
 RB proteins 261, 262, 265, 271
REVOLUTA (REV) 156, 223
RFL 108
RGA1 102
 Rho/Rac GTPase-related protein
 subfamily 31
Rhyniophyta 143
 rib meristem (RM) 58
 rib zone 20, 177, 178
 rice 24, 93, 100, 201, 258
 RKIP 110
 RNA interference 77
 RNA-binding protein 188
 RNase III-like domains 115
 root apical meristem (RAM) 2
 root embryonic ontogeny 279
 root hairs 290
 Rosaceae 175
 Rosette leaves 25
ROTUNDIFOLIA (ROT) 232
ROUGH SHEATH (RS)
 RS 1 23, 40, 225, 246
 RS 2 37, 70, 222, 223, 227, 247

Salvia 175
SCARECROW (SCR) 156, 288, 289
SCF 122
SCHIZOID (SHZ) 184, 186
sel 100
 sectorized leaves 21
SELF-PRUNING (SP) 110, 111, 151
SEPALATA (SEP) 123, 124, 128
 SEP1 97, 106, 114, 124
 SEP2 97, 106, 114, 124
 SEP3 97, 106, 114, 124
 serine-threonine 256
 protein kinase 257
 specific phosphatases 265
 sheath 225
 shoot apical meristem (SAM) 58, 215
SHOOTLESS (SHL) 24, 77, 78

SHOOTMERISTEMLESS (STM) 24-26, 28,
 30, 35-37, 41, 69, 70, 72, 73, 75, 76,
 78-81, 106, 119, 120, 146, 153, 155,
 163, 167, 179, 180, 183, 184, 195,
 219, 271, 272
SHOOTORGANIZATION (SHO) 35
SHORT INTEGUMENT1 (SINI) 61
SHORT VEGATATIVE PHASE (SVP) 96, 104
SHORT-ROOT (SHR) 288, 289
shz 186
 signal transduction pathway 157
Sinapis 105, 264
SKP1 78, 122
 snapdragon 21, 36, 176
 Solanaceae 175
 soybean 21
 S-phase 20, 254, 255, 257, 260, 261, 262,
 263, 266
Sphenophyta 143
 spike 92
SPINDLY (SPY) 96, 97, 113, 115
 spiral 172, 189, 199
 spiral phyllotaxis 192
Spirogyra 2
 spruce 20
SQUAMOSA (SQUA) 107, 109, 110, 118,
 185, 188
SQUAMOSA PROMOTER BINDING
 PROTEIN-LIKE 3 (SPL3) 96, 118
SQUAMOSA-PROMOTER BINDING
 PROTEINS (SBP1) 118
 stele 281
Stellaria 60, 82
 stem cells 4, 5, 31, 32
 STING/AUBERGINE 77
 storage granules 25
Strelitzia 145
Streptocarpus fanniniae 165
 stunted plant 1 265
 sunflower 38, 90, 173
 capitulum 174
SUPERMAN (SUP) 97, 106, 114, 115
SUPPRESSOR OF OVEREXPRESSED
 CONSTANS1 (SOC1) 105
 surgical experiments 216
suspensor 59, 64
 sympastic 47, 48
 sympodial 142, 145, 146, 158
sympodial (sym) 148, 150

³H-thymidine labeling 3, 5
 tangled 201, 231

- telome 214, 246, 247
telophase 260
tendriless (tl) 241, 242
TEOSINTE BRANCHED 1 (TBI) 162
TERMINAL EAR1 (TE1) 40, 185, 188, 219
TERMINAL FLOWER (TFL) 96, 151, 155
TFL1 96, 97, 106, 110, 111, 112, 116, 118, 119, 127
TFL2 96, 106, 111
tf1 tf2 double mutant 111
theory of the first available space 182
thorn apple 18
Three Amino acid Loop Extension (TALE) 22
TIMING OF CAB EXPRESSION1 (TOC1) 97, 99
tobacco 21, 146, 148, 163, 173, 258, 264, 267, 268
tomato 21, 89, 112, 146, 148, 150, 158, 173, 177, 194, 196, 197, 201, 202
ton1 39
tonneau/fass 201
TOPLESS (TPL) 66
tornado1 288
TOUSLED (TSL) 107, 122, 185
transition zone 19
TRANSPARENT TESTA GLABRA (TTG) 290
TRANSPORT INHIBITOR RESPONSE (TIR) 263
trichoblasts 282, 283, 290
tricussate 175
phyllotaxis 176
trifoliolate 238
2,3,5-Triiodobenzoic acid (TIBA) 193
Trimerophyta 143, 145
trn2 288
tryptophan monooxidase (iaaM) 161
iso 122
TSO1 107, 122
tunica 7, 17, 19, 39, 44, 58, 177, 182, 217, 270
corpus 7, 17, 43, 60
layers 17
TWIN 64
tnw1 64, 65
tnw2 64
two-component histidine kinases 265
ubiquitin 259
ubiquitin ligase 126
Ulva 2
umbel 90, 91
unicellular 2
UNIFOLIATA (UNI) 108, 242, 243
UNUSUAL FLORAL ORGANS (UFO) 26, 78, 97, 107, 115, 116, 122, 123, 125
upper leaf zone 227
valyl-tRNA synthetase 64
variegation 230
vascular cambial meristem (VCM) 2, 12
vascular strands 28
vernalization 12
VERNALIZATION (VRN)
VRN1 94, 97, 101, 102
VRN2 94, 97, 101
vesicle transport 63
VHID 156
Vinca 175, 205
WD Domains 122
Wee1 259, 264
WEREWOLF (WER) 290
wheat 24, 40, 92
whorled phyllotaxis 174, 175
WIGGUM (WIG) 107, 119, 121, 184, 185
Wolffia borealis 165, 167
WOODEN LEG (WOL) 289
WUSCHEL (WUS) 8, 9, 26, 32, 33, 72, 73, 79, 82, 106, 119, 120, 179, 183, 184, 186, 246, 272
WW domain 101
xylem 282
YABBY (YBY) 224, 247
YBY2 224
YBY3 71, 224
Zea mays 4, 16, 90, 260
ZEITLUPE (ZTL) 96, 97, 98, 99
zinc-finger protein 101, 106
Zea mays MADS box (ZMM)
ZMM8 110
ZMM14 110
ZmOCL 44
ZmOCL1 44
ZmOCL4 44
ZmOCL5 44
ZmRB 262
zonation 217
Zosterophyllophyta 143
zwill 184
ZWILLE/PINHEAD (ZLL/PNH) 27, 28, 107
zygote 59, 60